EFFECTS OF THE INTRAVENOUS ADMINISTRATION OF GLYCEROL SOLUTIONS TO ANIMALS AND MAN [1]

By HENRY A. SLOVITER with the technical assistance of RITA M. TIETZE

(From the Harrison Department of Surgical Research, School of Medicine, University of Pennsylvania, Philadelphia, Pa.)

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It has been found that human erythrocytes can be stored in the presence of glycerol at below-freezing temperatures for long periods of time (1) and that subsequently they can be successfully transfused (2). The development of a method (3) which permits the transfusion of thawed erythrocyte-glycerol mixtures, without completely removing the glycerol, has led to the present investigation, whose aim was to determine the effects of the intravenous administration of glycerol solutions and particularly to determine whether glycerol can be safely administered intravenously to human subjects.

The glycerol molecule is a part of the neutral fat (triglyceride) molecule and is, therefore, a substance which animals and humans normally metabolize. It is known that glycerol is converted by the liver to glycogen and other carbohydrates (4). Several studies have shown that large quantities of glycerol can be administered orally to experimental animals and man without the appearance of adverse effects (5–8). However, there have been reports that the parenteral administration of glycerol to experimental animals has been followed by hemoglobinuria (9–11), hypotension (5, 12), central nervous system disturbances (12, 13), and weight loss (14). In the only reported instance of the parenteral administration of glycerol to man, no toxic effects were noted after the administration of very small quantities via artery or vein (15). In most cases where toxic effects have been reported to follow the parenteral administration of glycerol, the toxic effects have been more frequent and more pronounced after subcutaneous, than after intravenous administration.

The present investigation deals with the intravenous administration of glycerol solutions to the rabbit, the dog and to man. The results of this investigation show that a considerable quantity of glycerol in a relatively dilute solution containing electrolyte can be administered intravenously to man without producing any noxious effects.

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MATERIALS AND METHODS

Glycerol solutions. Merck analytical reagent grade glycerol was used directly without further purification. Solutions containing approximately 5 per cent glycerol, 5 per cent glucose, and 0.9 per cent sodium chloride were used for the human infusion studies, because these are the approximate concentrations of these substances in the erythrocyte-glycerol mixture which is produced when a previously frozen erythrocyte-glycerol mixture is prepared for transfusion (3). This solution was prepared by adding 50 Gm. of glycerol to 1,000 ml. of sterile 5 per cent glucose in 0.9 per cent sodium chloride solution and then autoclaving the solution for 20 minutes at 15 lbs. steam pressure. For the experiments with rabbits and dogs, solutions of this same composition as well as others described below were used.

Rabbit experiments. Unanesthetized albino rabbits weighing between 2 and 3 Kg. were used. Solutions containing approximately 5 per cent glycerol in addition to electrolyte and glucose were infused via an ear vein. Rectal temperatures were measured before, during and after each infusion. Samples of urine voided during the course of the infusion, and the first urine voided after the infusion were collected in each case. In some cases, samples of urines which were voided on the following day were also collected. All urine samples were tested for the presence of hemoglobin by the benzidine test (16).

In two experiments, blood samples from an incised ear vein were collected in a tube containing heparin for the determination of plasma hemoglobin concentrations. Samples were obtained before infusion, when 10 per cent and 90 per cent of the solution had been infused, and 30 minutes and approximately 20 hours after the completion of the infusion. The blood samples were promptly centrifuged, plasma was removed and the hemoglobin concentration measured as described below.

In all experiments, the animals were carefully observed during the infusion period and for a short time thereafter for the appearance of muscle tremors, abnormal pupillary reactions or any behavior which might indicate central nervous system irritation.

Dog experiments. Dogs were anesthetized with pen-

tobarbital (35 mg. per Kg.) and kymographic recordings of respiration and arterial blood pressure (from a cannulated carotid or femoral artery) were made. Solutions of various glycerol concentrations were infused via a cannulated femoral vein. Urine samples were collected during the course of the experiment and were tested for hemoglobin.

To determine whether the effects observed might be due to actions on the central nervous system, glycerol solutions were injected directly into a carotid artery, both before and after cutting the vagus nerves. Injections were also made into the descending aorta via a polyethylene catheter which had been inserted into the left carotid artery and advanced down the aorta to about the level of the diaphragm.

Human experiments. Hospitalized patients who were in a stable state and showed no functional abnormalities of their cardiorespiratory, urinary or central nervous systems were infused intravenously with the glycerol solution. Nearly all the human subjects had malignant disease and were considered to have quite limited life expectancies. In each case, 1 L. of solution containing 50 Gm. of glycerol, 50 Gm. of glucose and 9 Gm. of sodium chloride was infused, except that in a few cases the salt was omitted. The duration of the infusion varied from three to six hours.

Immediately prior to infusion, pulse rate, respiratory rate, blood pressure, and oral temperature were measured. During the course of the infusion, pulse rate, respiratory rate and blood pressure were measured every 15 minutes for the first hour and thereafter every 30 minutes and again shortly after completion of the infusion. Temperature measurements were made at least twice during and once after completion of the infusion. The patients were carefully observed for any change in status, and were asked if they experienced any unusual sensations during the course of the infusion. A urine sample was obtained before the start of the infusion, and one or more samples were obtained during its course, and the first urine voided following the administration of the glycerol solution was collected. In a few cases, samples of urine voided on the morning following the infusion were collected. All urine samples were tested for hemoglobin by the benzidine test.

In four cases, venous blood samples were carefully drawn into syringes which had been wet with heparin solution. In each case a blood sample was obtained prior to infusion, another just before completion of the infusion, and a third sample seven hours after completion. The samples were promptly centrifuged, plasma was removed, and the hemoglobin concentration of each plasma sample was measured as described below.

Determination of hemoglobin in plasma. Oxyhemoglobin has a strong optical absorption maximum at 414 mμ, which conveniently permits the detection and estimation of low concentrations of hemoglobin. To correct for the variations among plasma samples in nonspecific optical absorption, due mostly to opalescence, optical densities were measured at 365 mμ, as well as at 414 mμ. It was found that the difference between the optical densities at 414 mμ and 365 mμ was a linear function of hemoglobin concentration in the range of 0 to 20 mg. hemoglobin per 100 ml. of plasma. A working curve was prepared by determining this difference for samples of plasma to which known quantities of hemoglobin had been added. This method was not exhaustively tested, and is not proposed at the present time as a generally applicable method. It may, in the presence of interfering substances, be subject to positive errors but is quite unlikely to be subject to appreciable negative errors. A few of the plasma samples were analyzed by both this spectrophotometric method and by a chemical method (17), and the results obtained by the two methods were in good agreement.

The measurements were carried out in the following manner. To 2.0 ml. of buffered (pH 7.4) saline solution was added 1.0 ml. of the plasma sample and the mixture was well mixed. Optical densities were then measured in a 10 mm. Corex cell at 414 mμ and 365 mμ using a Beckman model B spectrophotometer. From the differences in optical densities at these two wave lengths, the hemoglobin concentrations of the plasma samples obtained during and after the infusion were calculated and expressed as increases over that of the preinfusion plasma sample.

RESULTS

Rabbit experiments

Six experiments with unanesthetized rabbits were performed in which the solutions administered contained 5 Gm. each of glycerol and glucose and 0.9 Gm. sodium chloride per 100 ml. The quantities infused varied from 120 ml. to 175 ml. per rabbit, and were administered during periods varying from 60 to 90 minutes. No evidence of central nervous system irritation or any abnormal behavior was observed in any case. An elevation of rectal temperature of 1 to 2° F. occurred in two cases, but these returned to preinfusion levels within two hours after infusion. The rabbits urinated copiously and all urine specimens were found to be free of hemoglobin. The rabbits were normally active immediately following the fluid administration and remained so. There was no evidence of any harmful effect having been caused by the intravenous administration of the glycerol-containing solution.

In two additional experiments, 80 ml. and 100 ml., respectively, of the same 5 per cent glycerol solution were infused during a period of approximately one hour. Plasma samples were obtained, and their hemoglobin concentrations were determined as described above. In both rabbits, the

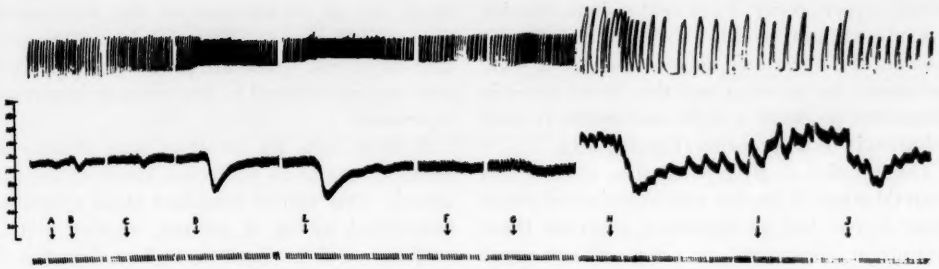

FIG. 1. BLOOD PRESSURE AND RESPIRATION OF 10.1 KG. DOG DURING INTRAVENOUS ADMINISTRATION OF GLYCEROL SOLUTIONS

A. Five per cent glycerol-5 per cent glucose-0.9 per cent NaCl solution at 20 ml. per minute.
B. Same solution as in A, 20 ml. in 15 seconds.
C. Twenty per cent glycerol-0.9 per cent NaCl solution, 10 ml. in 10 seconds.
D. Same solution as in C, 20 ml. in 15 seconds.
E. Fifty per cent glycerol-0.9 NaCl solution, 10 ml. in 10 seconds.
F. Nine-tenths per cent NaCl solution, 20 ml. in 15 seconds.
G. Five per cent NaCl solution, 20 ml. in 20 seconds.

Vagus nerves were cut between G and H.

H. Fifty per cent glycerol-0.9 per cent NaCl solution, 20 ml. in 20 seconds.
I. Same solution as in H, 4 ml. into carotid artery.
J. Same solution as in H, 10 ml. into descending aorta.

plasma hemoglobin concentrations of the samples taken near the end and shortly after infusion were higher than the preinfusion level by 5 mg. per 100 ml. The samples taken near the beginning of the infusion and on the following day had hemoglobin concentrations which were not measurably different from the preinfusion levels (differences not greater than 2 mg. per 100 ml.).

Dog experiments

Three experiments with dogs under pentobarbital anesthesia were carried out. In one case, 50 ml. of the 5 per cent glycerol-5 per cent glucose-0.9 per cent sodium chloride solution was injected intravenously during a 15 minute period prior to administration of pentobarbital. No signs of central nervous system irritation or abnormal behavior were observed following this injection. The infusion in a 12 Kg. anesthestized dog of a total of 200 ml. of this solution, during a 50 minute period caused no change in blood pressure or respiration. The administration of this solution at the rather rapid rate of 20 ml. per minute had no significant effect on blood pressure or respiration. The infusion of 20 ml. of this solution in 15 seconds caused a small drop (about 16 mm.) in blood pressure which returned to its previous level in less than one minute. Infusion of 0.9 per cent sodium chloride solution at this same rapid rate had no effect on blood pressure. These effects are shown in Figure 1, items A, B and F.

The infusion of 20 per cent glycerol-0.9 per cent sodium chloride solution at the rate of about 6 ml. per minute caused no change in blood pressure or respiration. Giving 10 ml. in 10 seconds caused a small transient drop (10 mm.) in blood pressure and infusion of 20 ml. in 15 seconds caused a sharp drop (50 mm.) in blood pressure which returned to its previous level in about one minute (Figure 1, C and D). There was also an increase in respiratory rate. Similar effects of somewhat smaller magnitude occurred when 20 per cent glycerol in water was infused. When 50 per cent glycerol-0.9 per cent sodium chloride solution was infused at the rate of 2 ml. per minute, small fluctuations in blood pressure were observed and when 10 ml. was infused in 10 seconds, blood pressure rapidly dropped 60 mm., respiratory rate increased markedly, and both returned to previous levels in about two minutes (Figure 1, E).

To determine to what extent the effects observed with these glycerol solutions might be due

to their hypertonicity, 5 per cent sodium chloride was infused intravenously. It was found that infusion of this hypertonic saline at the rate of 4 ml. per minute had no effect, and that 20 ml. given in 20 seconds produced a slight and relatively slow reduction in blood pressure (Figure 1, G).

The injection of glycerol solution, directly into a carotid artery of the dog with vagus nerves either intact or cut, had no significant effect on blood pressure or respiration. In a dog with cut vagus nerves, the intravenous infusion of a 50 per cent glycerol solution produced a sharp drop in blood pressure, similar to that produced in the dog with intact vagus nerves except that in the former case, the return of the blood pressure to its previous level was considerably slower (Figure 1, H and I).

In one dog with vagus nerves cut, the injection of glycerol solution was made into the descending aorta, via a polyethylene catheter which had been inserted in the left carotid artery and advanced till its orifice was at about the level of the diaphragm. The injection here of 10 ml. of 50 per cent glycerol in 0.9 per cent sodium chloride solution produced a sharp drop in blood pressure which slowly returned toward the previous level (Figure 1, J).

Urine specimens collected at various times during the experiments with the dogs were all found to contain no hemoglobin.

Human experiments

Each of a series of 12 patients was given intravenously 50 Gm. of glycerol in a liter of solution. The observations made on these patients, both prior to and during the course of the infusions, are listed in Table I. It can be seen that, in most cases, blood pressure was little changed from the preinfusion level during the course of the infusion. In a few cases, there was a small rise in blood pressure, but no more than might be expected from the relatively rapid intravenous administration of any considerable amount of fluid to a group of older patients. In no case was there any change in cardiac or respiratory rate of appreciable magnitude. Body temperature showed no appreciable change, which might have been due to a reaction to the glycerol, for in no case was there an elevation of temperature of more than one degree. A small rise in body temperature might be expected as a result of the metabolism of the administered glycerol and glucose. Occasional small temperature elevations, which are probably due to pyrogens, are not unusual in any series of intravenous injections.

In most cases the infusions were entirely uneventful, insofar as subjective reactions are concerned. One patient remarked about a transient generalized feeling of warmth, another of nasal stuffiness of short duration, and another that her legs felt cold for a few minutes. There was no occurrence of headache, visual symptoms or dizziness. No cardiac irregularities were observed. That the infusions were completed without serious incident is indicated by the fact that in no case was it necessary to discontinue or interrupt the infusion before its completion. There was no complaint of pain at the site of injection, and no thrombosis of the veins which were used was noted.

In most cases, a moderate diuresis resulted from the infusions. In each case, one or more urine specimens were collected both during and after the infusion, and in some cases on the following day. All urine specimens in all cases were found to contain no hemoglobin.

Four additional patients were infused with a liter of solution containing 50 Gm. of glycerol in the same manner as were the other patients, except that samples were obtained for the determination of plasma hemoglobin concentrations. Two of the patients received solutions containing the glucose (5 per cent) and sodium chloride (0.9 per cent) as well as the glycerol, while in the other two cases, the sodium chloride was omitted. In all four cases, the subjective and objective reactions of the patients to the infusions were not significantly different from those observed with the other patients. The plasma hemoglobin concentrations of the samples taken just before completion of the infusions were higher than the preinfusion values by 3 and 8 mg. per 100 ml. in the two cases where the solutions infused contained salt, and by 4 and 10 mg. per 100 ml. in the two cases where the solutions did not contain salt. In all four cases, the plasma hemoglobin concentrations of the samples obtained seven hours after the infusion were not measurably different from the preinfusion levels.

In Patient 3, who had a pneumonitis and whose

TABLE I

Intravenous administration of glycerol solutions to human subjects*

Patient, description and diagnosis	Duration of infusion (*hours*)	Blood pressure		Temperature (° F.)		Pulse rate		Respiratory rate	
		Before infusion	During infusion	Before	During	Before	During	Before	During
1. 55 yr. male 95 lbs., parotid carcinoma	6	90/70	98/70–105/75	100.0	98.8–100.0	104	96–104	20	18–20
2. 75 yr. female 110 lbs., mammary carcinoma	5	145/85	145/80–155/90	99.0	99.0–100.0	80	76–88	20	20–22
3. 66 yr. male 120 lbs., pancreatic carcinoma	6	120/80	120/70–140/80			110	100–115	30	26–30
4. 41 yr. female 118 lbs., colon carcinoma	6	124/78	114/64–124/78			104	96–108	20	20–24
5. 75 yr. female 105 lbs., pancreatic carcinoma	4	116/60	108/54–116/60	98.6	98.6–98.6	60	56–80	20	18–20
6. 39 yr. female 110 lbs., mammary carcinoma	6	118/90	110/78–130/80	99.0	98.8–100.0	84	80–100	20	20–28
7. 56 yr. female 83 lbs., stomach carcinoma	4	110/60	108/66–116/60	98.4	98.6–98.8	92	92–100	20	20–22
8. 60 yr. female 170 lbs., malignant melanoma	3¼	130/70	128/70–146/88	98.8	99.0–99.4	68	72–80	20	18–22
9. 66 yr. female 135 lbs., mammary carcinoma	4	90/70	88/70–116/74	100.2 (R)	100–101.2	112	100–116	28	26–28
10. 37 yr. female 147 lbs., mammary carcinoma	3¼	108/70	108/70–118/70	99.4	98.6–99.8	112	100–104	20	18–20
11. 73 yr. female 110 lbs., mammary carcinoma	3¾	145/90	148/88–164/100	98.8	98.8–98.8	88	78–82	18	18–20
12. 68 yr. female mammary carcinoma	3½	138/70	120/64–138/70	98.0	98.2–98.4	100	96–104	20	20–22

* In all cases except No. 9 one L. of solution which contained 50 Gm. of glycerol, 9.0 Gm. of sodium chloride and 50 Gm. of glucose was infused. In case No. 9 the sodium chloride was omitted.

chest was full of coarse râles, there was significant clearing of his lungs after the infusion, as judged by auscultation. This observation is noted as being of possible significance, only because of a report that glycerol has a bronchodilating action (18).

DISCUSSION

The results of the present study indicate that considerable amounts of glycerol in moderately dilute solution can be safely administered intravenously to animals and man. In the present study, the highest total quantity administered to man in

terms of body weight is 1.2 Gm. per Kg. For the rabbit, this value was higher than 2 Gm. per Kg. and for the dog, higher than 3 Gm. per Kg. These findings are not entirely in agreement with those of some previous studies in experimental animals. Some of the differences in results and conclusions are probably due to differences in the concentration and composition of the solutions administered, and to differences in rate, and the particular parenteral route of administration which were employed. It appears that most of the toxic effects previously reported were observed in cases where undiluted or very concentrated glycerol was injected. For example, Solomidès (14) reported that 1.0 Gm. per Kg. of undiluted glycerol given intravenously to rabbits was uniformly fatal, but that the same quantity of glycerol previously diluted with an equal quantity of saline solution produced neither mortality nor morbidity. It would seem reasonable to conclude that, in this case, the toxic effects reported were due to the osmotic disturbances which the undiluted glycerol caused rather than to an inherent toxicity of the glycerol molecule. The data reported in the review paper by Deichmann (19) also suggest that severe dehydration and osmotic disturbances were very likely responsible for the noxious effects observed.

Cameron and Finckh (9) reported that in the rat, moderate intravenous doses of 50 per cent glycerol solution produced some hemolysis, and that large doses caused convulsions by dehydration. However, they found that large intravenous doses of a 20 per cent solution caused no hemolytic effects. They also reported considerable species differences in the occurrence of hemolysis; for example, in the rabbit they found no hemoglobinuria, even after intravenous injection of undiluted glycerol. These species differences may be related to rather large differences in the permeability to glycerol of the erythrocytes of the different species. That glycerol itself is not a hemolytic agent is indicated by the fact that large amounts of glycerol can be added to blood *in vitro* without producing any lysis as long as the ionic strength of the mixture is not markedly decreased (1). In the present study, the uniform absence of hemoglobinuria during and after the infusion of glycerol solutions in all three species demonstrates that no considerable amount of intravascular hemolysis occurs. However, hemoglobinuria is a relatively insensitive test for the occurrence of hemolysis, because of the renal threshold for hemoglobin. It is, however, evident, from the results of the direct measurement of plasma hemoglobin concentrations, during and after the infusion of glycerol solutions, that no more than a very slight amount of hemolysis occurs. Plasma hemoglobin concentrations in normal individuals are of the order of 5 mg. per 100 ml. (20). The increases in plasma hemoglobin concentration which were noted in the present study were in no case greater than 10 mg. per 100 ml., either during or after the administration of glycerol solutions. Increases of this magnitude might well occur during the infusion of more conventional intravenous fluids. There appears to be no published report on the effect of the routine administration of intravenous fluids on plasma hemoglobin levels.

Previous studies have disclosed that hemolytic effects were frequent after subcutaneous injection of glycerol solutions, but were absent or infrequent after intravenous administration. In nearly all cases, the solutions injected were markedly hypertonic. This difference in the frequency of occurrence of hemolysis, between subcutaneous and intravenous administration, might well be due to the fact that the osmotic effects of hypertonic solutions are minimal when given intravenously, because of the rapid dilution by the circulating blood. There is no evidence that glycerol reacts with the subcutaneous or other tissues to give rise to a hemolytic substance, but this possibility is not excluded. It should be remembered that the unintentional perivascular infiltration of a glycerol solution may produce some undesirable effects.

In the present study, no significant effects on blood pressure were observed in man or dog, except that in the dog very rapid intravenous injection of concentrated solutions (*e.g.*, 10 ml. in 10 seconds of 50 per cent solution) produced transient hypotension. This is in contrast to the statement by Johnson, Carlson, and Johnson (5) that in the dog, "even a small dose of glycerol intravenously produces a transient fall in arterial pressure." It is possible that the hypotensive effects observed by these authors may have been due to an impurity in the glycerol which was used. In the present study, the transient hypotension produced by the rapid intravenous injection of concentrated glycerol solution seems most likely to

be due to peripheral vasodilation. Glycerol might be expected to have a vasodilating action because of its chemical similarity to ethanol. That the hypotension is not due to an effect on the central nervous system is indicated by the absence of hypotension when glycerol solution was injected into a carotid artery. Reflex vagal slowing of the heart could not have been the cause of the hypotension, since the hypotensive effect persisted after cutting the vagus nerves. A depression of myocardial contraction by glycerol is not excluded, but seems unlikely because injection of concentrated glycerol solution into the descending aorta produced a prompt fall in blood pressure. Another possible cause of hypotension when intravascular hemolysis occurs is the release of nucleotides from the erythrocytes. It has been shown that these nucleotides have a potent hypotensive action (21). In the present study, no significant degree of intravascular hemolysis occurred, and thus it is very unlikely that the transient hypotension was due to release of nucleotides.

The previously reported occurrence of convulsions and other manifestations of central nervous system dysfunction (9, 12) were also quite possibly due to the severe osmotic disturbance, produced by the injection of quite concentrated solutions of glycerol. In the present study, where the tonicity of the administered solutions was not high enough to produce appreciable osmotic derangement, no signs of central nervous system irritation were observed. The reported occurrence of central nervous system irritation one or more days after injection of glycerol seems to be best explained by the tissue dehydration and electrolyte disturbance resulting from the diuresis which was produced (13).

The demonstration that glycerol can be safely administered intravenously in considerable quantity opens the way to the possible use of this substance as an intravenous nutritional agent. That glycerol is rapidly metabolized and gives rise to blood glucose is indicated by the findings that intraperitoneal injections of glycerol solutions in rabbits (22) and oral administration of glycerol to man (6) cause hyperglycemia. Furthermore, insulin convulsions in the rat were promptly relieved by the intraperitoneal injection of glycerol solutions (23). It has also been shown that glycerol solutions have an antiketogenic effect when injected intraperitoneally in starved ketonemic rats (24). It is possible that glycerol is more efficiently utilized than is glucose, by individuals suffering from diabetes or other metabolic diseases. Glycerol has recently been used to stabilize fat emulsions, which are intended as intravenous nutritional preparations (25). Glycerol may also be a useful solvent in preparations of drugs for intravenous administration.

SUMMARY

The intravenous administration of solutions containing 5 per cent glycerol to experimental animals and to man has been found to cause no toxic or otherwise undesirable effects. Human subjects have been given intravenously 50 Gm. of glycerol in a liter of solution which contained, in addition to the glycerol, 50 Gm. of glucose and 9.0 Gm. of sodium chloride. No disturbances of cardiorespiratory or central nervous system function were observed, and no significant hemolytic effects occurred.

The previously reported toxic effects of parenterally administered glycerol have not been observed, and there are indications that these toxic effects were due to the osmotic disturbances produced by the injection of glycerol solutions of high concentration. In the dog, the rapid injection of concentrated solutions of glycerol produced a transient drop in blood pressure, and this was probably due to a peripheral vasodilating effect.

It is suggested that intravenously administered glycerol may be a useful nutritional agent.

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COMPARISON OF RESPONSE TO INTRAVENOUSLY ADMINISTERED SODIUM TOLBUTAMIDE IN MILD DIABETIC AND NONDIABETIC SUBJECTS [1, 2]

By ROGER H. UNGER and LEONARD L. MADISON

(From the Departments of Internal Medicine, University of Texas Southwestern Medical School, and Veterans Hospital, Dallas, Texas)

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The hypoglycemic response of moderately severe diabetic patients to an orally administered test dose of tolbutamide has been shown by Mirsky, Diengott, and Dolger (1) to differ strikingly from that of nondiabetic subjects. Indirect evidence of a correlation between tolbutamide responsiveness and pancreatic insulin content (1), coupled with experimental support for the view that the drug stimulates endogenous secretion of insulin (2–6), suggest that this difference may be of more than empirical interest. The purpose of the following study was to compare response to intravenously administered tolbutamide in nondiabetic and mild diabetic subjects, including those with a normal and near-normal fasting blood glucose level.

MATERIALS AND METHODS

One hundred nondiabetic controls were selected from the medical, surgical, and otolaryngological wards of the Dallas Veterans Hospital (85 subjects), and from among medical students (15 subjects). The nondiabetic status of all control subjects was established by means of history, physical examination, and a negative oral glucose tolerance test, interpreted according to the criteria of the American Diabetes Association (7). Seventy-nine mild, stable, diabetic patients, *none* of whom required insulin therapy, were selected from the Parkland Memorial Hospital Metabolic Clinic, from the wards of the Dallas Veterans Hospital, and from among subjects discovered by the Diabetes Detection Unit of the Dallas City Health Department. Diabetic subjects were subdivided according to their fasting blood glucose level on the morning of the test. Thirty-four patients had fasting hyperglycemia of diagnostic proportions (115 to 180 mg. per cent) and 45 had normal or slightly elevated fasting blood glucose levels (below 115 mg. per cent). Twenty-five of the latter had levels below 100 mg. per cent. The diabetic status of the latter group was established by means of three hour oral glucose tolerance tests. Because of the nonspecificity of this test, as usually interpreted, in the borderline zone (8, 9), the following stringent diagnostic criteria for diabetes were employed: peak blood glucose level, 200 mg. per cent or higher; two hour level, 170 mg. per cent or higher; and three hour level, 135 mg. per cent or higher.

All subjects were requested to adhere to a diet containing 300 Gm. of carbohydrate daily for at least three days before the test. After an overnight fast, and the withdrawal of a fasting blood specimen, 1 Gm. of sodium tolbutamide,[3] diluted in 11 ml. of distilled water, was injected intravenously at a constant rate over a two minute period. Blood specimens were drawn 20, 30, 40, 60, and, in some instances, 90 and 120 minutes after the midpoint of the injection period. Blood glucose concentration was determined in duplicate by the Somogyi-Nelson technique (10). In our laboratory the standard deviation of the replicate determinations included in this study was 1.10.

RESULTS

The typical blood sugar response, exhibited by nondiabetic subjects to intravenously administered sodium tolbutamide, simulated the pattern usually obtained after intravenous injection of insulin. Glucose concentration declined rapidly to a nadir in 20 to 50 minutes after injection, following which a rebound towards normal usually occurred (Figure 1). At 20 minutes after the injection, the blood glucose level of nondiabetic subjects had declined to an average of 60 per cent of the pretest value (S. D., ± 14.2), and the range was from 85 to 9 per cent. Thirty minutes after injection, the mean fall was to 51 per cent of the pretest level (S. D., ± 14.8), with a range of from 82 to 6 per cent. At 40 minutes, at which time rebounding values became increasingly prevalent, the mean percentage was 55.6 (S. D., ± 11.9), with a range of from 91 to 22 per cent. At 60 minutes the mean was 72 per cent (S. D., ± 12.1), and the range from 104 to 42 per cent (Figure 1, 2).

[1] This work was supported by a grant from the Upjohn Company, Kalamazoo, Michigan.

[2] Presented in part at the annual meeting of the American Federation of Clinical Research, May, 1957.

[3] Sodium tolbutamide was generously supplied by Dr. C. J. O'Donovan, Upjohn Company, Kalamazoo, Michigan.

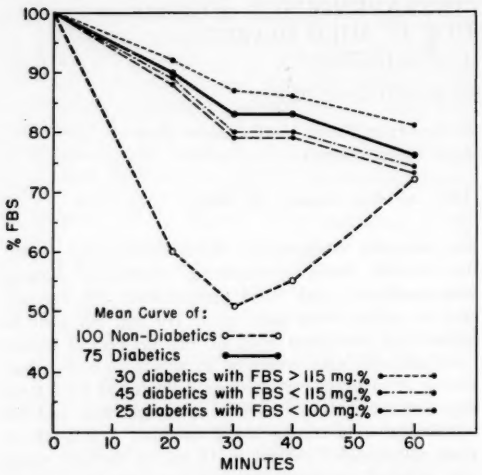

FIG. 1. MEAN TOLBUTAMIDE RESPONSE CURVES OF ALL BUT FIVE OF THE SUBJECTS STUDIED ILLUSTRATING THE BLOOD GLUCOSE RESPONSES TYPICAL OF NONDIABETICS AND OF MILD DIABETICS OF VARYING DEGREES OF SEVERITY

The curve of 45 diabetics with fasting blood specimen < 115 mg. per cent includes the 25 with fasting blood specimen < 100 mg. per cent whose mean curve is also shown separately. Five of the 79 diabetics in this study are not represented in this figure, but their omission does not perceptibly affect the results.

In striking contrast to the response of nondiabetics, diabetic subjects exhibited a more gradual decline which sloped towards a nadir somewhere beyond the one hour specimen (Figure 1). Twenty minutes after injection, the blood glucose level of the diabetic group had fallen to a mean of only 90 per cent of the pretest level (S. D., ± 7.4), with a range of from 108 to 61 per cent. At 30 minutes, the mean percentage of pretest values was 83 per cent (S. D., ± 8.7) with a range of from 105 to 49 per cent. At 40 minutes after injection, the mean was 83 per cent (S. D., ± 9.8), with a range of from 107 to 53 per cent. At 60 minutes, the mean was 77 per cent (S. D., ± 10.9), with a range of from 129 to 49 per cent (Figure 1).

A separate analysis was made of the 45 diabetics with a fasting blood glucose level of less than 115 mg. per cent, and of 25 diabetics with a fasting level below 100 mg. per cent (Figure 1). The hypoglycemic response of the milder diabetics is somewhat more rapid than those whose fasting blood glucose levels exceed 115 mg. per cent. Nevertheless, the mean tolbutamide response curve of each of these groups is shown to parallel closely the mean response curve of the more severe diabetics, and to differ strikingly from the mean response curve of the nondiabetic group.

In Figure 3, the individual response curves of 12 nondiabetic controls are compared with those of 12 diabetic subjects whose fasting blood glucose levels were in a similarly normal range. The precipitous decline in the blood glucose level of the nondiabetics, followed typically by a rebound toward the pretest level, is in marked contrast to the more gradual and often irregular slope exhibited by the mild diabetics.

In Figure 2, the individual blood glucose levels of all diabetic and nondiabetic subjects, expressed as per cent of the pretest value, are recorded at 20, 30, 40, 60, and in a few instances, at 90 and 120 minutes after the tolbutamide injection. Separation of the two groups is maximal at 20 and 30 minutes, at which time the blood glucose level of nondiabetics approaches its nadir. Overlap between groups becomes increasingly prevalent thereafter, as the blood glucose level of the nondiabetics returns towards its pretest value, while that of the diabetics continues to decline. At 20 minutes after the injection of tolbutamide, the blood glucose level of 96 per cent of nondiabetics has declined to below 84 per cent of the pretest value, and in 94 per cent, has fallen to below 80 per cent of pretest level. In contrast, the blood glucose level of 94 per cent of the diabetic group remained at 84 per cent of the pretest level or higher, and in 95 per cent, remained at 80 per cent or more.

At 30 minutes after the tolbutamide injection, the blood glucose level of 99 per cent of the nondiabetics fell below 77 per cent of the pretest value (Figure 2). However, in 10 per cent of diabetic patients, declines to 73 per cent of the pretest level or less are noted.

DISCUSSION

The foregoing data indicate that the rate of hypoglycemic response of nondiabetic subjects to intravenously administered sodium tolbutamide differs from that of mild stable diabetics. While precise interpretation of this difference must await final elucidation of the mechanism of sulfonylurea-

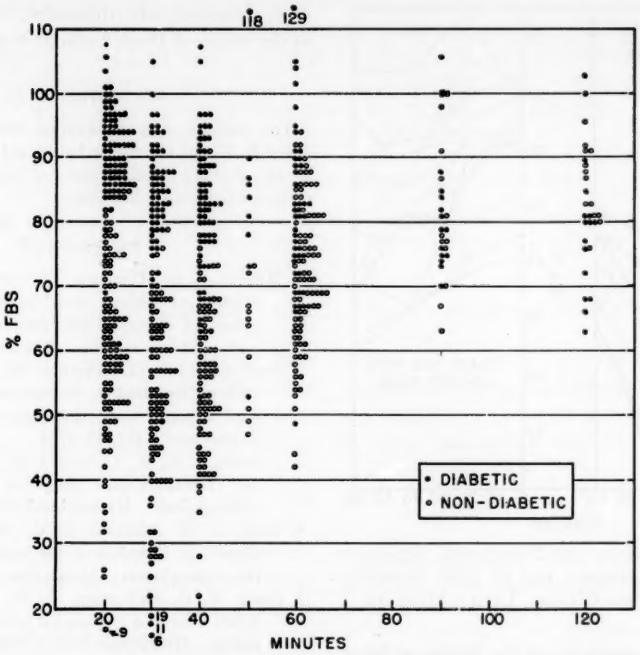

FIG. 2. THE TOLBUTAMIDE RESPONSE TESTS OF 100 NONDIABETIC AND 79 MILDLY DIABETIC SUBJECTS ARE RECORDED

Blood glucose levels are expressed as per cent of the fasting concentration. Separation of the two groups is maximal at 20 and 30 minutes.

induced hypoglycemia, speculation as to its significance is of interest.

Although this study in no sense contributes to clarification of the problem of its mechanism of action, the results are in harmony with the concept that tolbutamide enhances the release of insulin from the beta cells (2–6). The resemblance between the nondiabetic tolbutamide response curve and the normal insulin tolerance curve is compatible with a release of stored insulin from the beta cells (1). If this were the case, the more gradual hypoglycemic response to tolbutamide exhibited by diabetic subjects could be ascribed either to diminished insulin stores, or to decreased rate of insulin release in response to betacytotropic stimulation, although extrapancreatic interference with insulin degradation is also possible. The apparent inverse correlation between the rate of hypoglycemic response and the fasting blood glucose concentration noted in the diabetic group (Figure 1) may be a quantitative expression of the severity of the disease, or merely a nonspecific function of the pretest blood glucose level.

There is, however, experimental evidence that tolbutamide potentiates the action of exogenous insulin in totally depancreatized animals (11–13). The results of the present study can also be explained on the basis of potentiation by tolbutamide of independently secreted endogenous insulin.

Validation of either of these theories of sulfonylurea action would provide a rational basis for the observed differences in tolbutamide response. In this event, the intravenous tolbutamide response test would constitute an index of beta cell function, which might prove useful as a new parameter for the diagnosis of mild diabetes. Attention is called to the fact that the blood glucose concentration at 20 and 30 minutes, expressed as per cent of pretest level, correctly segregated diabetic patients from nondiabetics in approximately 95 per cent of cases; this includes the mildest dia-

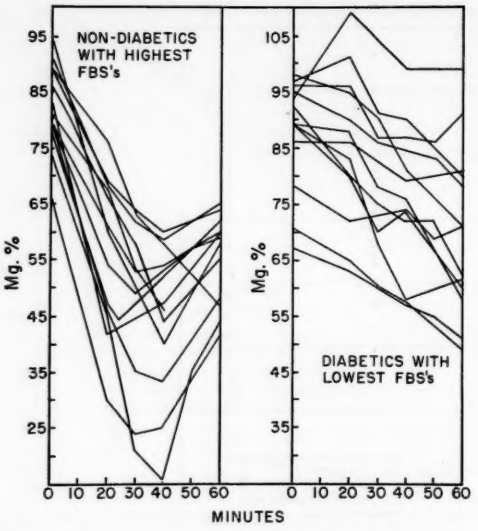

FIG. 3. COMPARISON OF THE TOLBUTAMIDE RESPONSE CURVES OF 13 NONDIABETICS AND 13 MILD DIABETICS WHOSE FASTING BLOOD GLUCOSE LEVELS WERE IN A SIMILAR RANGE

The rapidity and magnitude of the decline of blood glucose levels which characterize the nondiabetic group are in contrast to the more gradual and occasionally irregular responses of the diabetics.

betics, whose fasting blood glucose levels were in a normal or near-normal range. Since this record compares favorably with other routine clinical tests now employed in the diagnosis of mild diabetes (8, 9), further evaluation of the tolbutamide response test as a diagnostic procedure seems warranted.

SUMMARY

Intravenous sodium tolbutamide response tests were performed in 100 nondiabetic and 79 mild, stable diabetic subjects, and blood glucose concentration determined at 20 minute intervals for up to two hours thereafter. Whereas the blood glucose levels of nondiabetics fell rapidly, reaching a nadir between 20 and 40 minutes after injection, diabetics, including those with a normal or near-normal fasting blood glucose value, exhibited a more gradual decline. Possible explanations for this striking difference in tolbutamide response are discussed, and the practical implications of these findings considered.

ACKNOWLEDGMENTS

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THE PHYSIOLOGIC SIGNIFICANCE OF THE SECRETION OF ENDOGENOUS INSULIN INTO THE PORTAL CIRCULATION. I. COMPARISON OF THE EFFECTS OF GLUCAGON-FREE INSULIN ADMINISTERED VIA THE PORTAL VEIN AND VIA A PERIPHERAL VEIN ON THE MAGNITUDE OF HYPOGLYCEMIA AND PERIPHERAL GLUCOSE UTILIZATION [1]

By LEONARD L. MADISON AND ROGER H. UNGER

(From the Department of Medicine, University of Texas Southwestern Medical School, Dallas, Texas, and the Department of Medicine, Veterans Administration Hospital, Dallas, Texas)

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The metabolic consequences of exogenous insulin which is administered parenterally may differ from those of the endogenously secreted hormone for at least two reasons: exogenous insulin may be altered in its commercial preparation; its activity may be different because it enters the body through the systemic circulation. Under physiologic conditions, insulin is secreted by the pancreatic betacytotropic cells into the branches of the portal circulation, traversing the liver prior to entry into the systemic circulation where it is bound in a variety of tissues; this binding is apparently intimately associated with insulin activity (1–4). Theoretically, insulin secreted into the portal vein, because it passes directly into the liver, may have a metabolic effect different from insulin administered peripherally: all of the insulin reaches the liver, and may therefore increase the hepatic effect and decrease the peripheral effect (qualitative difference); part of the insulin may be destroyed by hepatic insulinase reducing the net amount available (quantitative difference); finally, there may be no significant physiologic alteration by this initial passage through the liver.

The purpose of this study was to determine whether the *initial passage* of insulin through the liver altered its subsequent metabolic activity qualitatively or quantitatively by comparing the effect of glucagon-free insulin [2] administered via the portal and via a peripheral vein on the magnitude of the hypoglycemia and the magnitude of the change in peripheral glucose utilization. The arteriovenous (A–V) glucose difference and the arteriovenous glucose difference/arterial glucose concentration (A–V)/A were used as the parameters for measuring changes in peripheral glucose utilization (5, 6).

METHODS

Sixteen experiments were performed on eight healthy adult dogs, housed in individual runs and maintained on a diet of constant composition for at least two weeks prior to each experiment. All procedures were performed in an airconditioned constant temperature room on dogs in the postabsorptive state. Sodium pentobarbital (Nembutal®, 25 mg. per Kg.) was used for anesthesia. Two experiments were conducted in random order on each dog: during one, glucagon-free insulin (0.07 U per Kg.) was administered over a two minute period into a large tributary of the portal vein, and during the other, the same amount was given into a peripheral vein in the foreleg. In those instances when insulin was given via a peripheral vein, a sham operation was performed and saline injected into a tributary of the portal vein while the dog was simultaneously receiving insulin in a foreleg vein. This additional control, sham operation was necessary since other data from our laboratory indicated that one of the acute effects of operation was an augmentation of peripheral glucose utilization.

Arterial and venous blood samples were drawn simultaneously from the femoral artery on one side, and from the contralateral femoral vein by means of indwelling Cournand needles. About 30 minutes after the operation was completed, two control blood samples were obtained at 10 minute intervals prior to insulin injection, and thereafter usually at 5 minute intervals for the first 30 minutes, at 10 minute intervals for the following 30 minutes and at 15 minute intervals for the ensuing 30 to 60 minutes.

To prevent glycolysis, blood samples were collected in chilled tubes containing sodium fluoride. Blood glucose was determined in duplicate by the Somogyi copper iodometric method on 5 ml. of blood filtrate prepared immediately from 2 ml. blood samples, thereby providing a

[1] This work was supported in part by a grant from The Upjohn Company.

[2] The supply of glucagon-free insulin was furnished by Dr. W. R. Kirtley of the Eli Lilly Company, Indianapolis, Indiana.

TABLE I

Changes in femoral arterial and venous glucose concentrations after glucagon-free insulin (0.07 U per Kg.) administered into a foreleg vein

Dog		Control		Time after insulin administration													
		0–10	0	5	10	15	20	25	30	40	50	60	70	80	90	105	120	135
1*	A‡	93.7	96.0	90.8	82.4	66.0	56.0		59.0	71.3	79.0	82.0	86.8	91.0	87.6	89.9	88.8	93.9
	V§	92.3	96.2	86.2	78.6	60.0	48.0		47.8	59.0	61.0	69.0	68.1	75.0	71.4	75.0	81.4	84.0
	A-V‖	1.4	−0.2	4.6	3.8	6.0	8.0		11.2	12.3	18.0	13.0	18.7	16.0	16.2	14.9	7.4	9.9
	A-V/A¶	0.014	−0.002	0.050	0.046	0.090	0.142		0.189	0.172	0.227	0.151	0.215	0.175	0.184	0.165	0.083	0.105
6*	A	74.0	74.6	76.0	61.0	50.4	47.4		39.9	53.0	61.4	61.4	65.6	68.3	65.6	66.7	70.0	78.9
	V	72.4	72.8	70.8	56.4	46.8	40.7		35.0	43.3	53.2	53.2	55.6	58.2	54.8	61.7	67.0	76.7
	A-V	1.6	1.8	5.2	4.6	3.6	6.7		4.9	9.1	8.2	10.8	10.0	10.1	10.8	5.0	3.0	2.2
	A-V/A	0.021	0.024	0.068	0.075	0.071	0.141		0.122	0.171	0.133	0.168	0.152	0.147	0.164	0.074	0.042	0.027
5*	A	82.0	78.0	78.2		70.1	67.2		72.8	78.0	81.0	82.0	85.0	83.6	85.6	82.7	87.2	87.8
	V	79.0	75.5	74.5		62.3	55.4		57.3	64.2	66.0	69.6	73.2	75.7	76.1	75.0	78.0	79.8
	A-V	3.0	2.5	3.7		7.8	11.8		15.5	13.8	15.0	12.4	11.8	7.9	9.5	7.7	9.2	8.0
	A-V/A	0.036	0.033	0.047		0.111	0.175		0.212	0.176	0.185	0.151	0.138	0.094	0.110	0.093	0.105	0.091
8*	A	85.6	88.1	85.9	74.0	61.6	56.8		62.2	75.0	79.8	82.7	83.2	88.9	88.1	89.2		
	V	85.0	88.1	85.6	69.0	60.2	53.0		52.2	67.0	72.2	70.3	75.4	81.0	82.7	84.5		
	A-V	0.6	0.0	0.3	5.0	1.4	3.8		10.0	8.0	7.6	12.4	7.8	7.9	5.4	4.7		
	A-V/A	0.007	0.000	0.003	0.067	0.022	0.066		0.160	0.106	0.095	0.149	0.094	0.089	0.061	0.052		
3*	A	79.8	80.0	77.3	67.2	58.0	54.4		68.2	76.0	77.3	80.2	82.7	81.4	75.6	79.2	79.8	
	V	79.0	80.2	77.0	61.0	52.9	46.5		58.6	68.2	71.4	73.3	78.2	75.0	72.6	74.0	78.0	
	A-V	0.8	0.0	0.3	6.2	5.1	7.9		9.6	7.8	5.9	6.9	4.5	6.4	3.0	5.2	1.8	
	A-V/A	0.010	0.000	0.003	0.092	0.087	0.140		0.140	0.102	0.076	0.086	0.054	0.078	0.039	0.065	0.022	
4*	A		88.3			56.2	51.4	56.5	72.2	76.0	82.8	81.5		87.8	87.2	98.0		
	V		86.7			53.3	48.8	50.0	61.8	70.4	77.0	77.0		84.6	82.0	82.6		
	A-V		1.6			2.9	2.6	6.5	10.4	5.6	5.8	4.5		3.2	5.2	5.4		
	A-V/A		0.018			0.051	0.050	0.115	0.144	0.074	0.070	0.055		0.036	0.059	0.061		
2*	A	88.4	90.7	89.7	82.4	73.0	63.6	55.4	49.7	47.2	50.8	57.8	67.0	73.4	76.2			
	V	86.7	90.6	88.8	81.2	72.0	63.0	55.3	49.4	45.4	48.2	55.0	63.2	69.0	71.6			
	A-V	1.7	0.1	0.9	1.2	1.0	0.6	0.1	0.3	1.8	2.6	2.8	3.8	4.4	4.6			
	A-V/A	0.019	0.001	0.010	0.014	0.013	0.009	0.002	0.006	0.036	0.052	0.048	0.057	0.059	0.060			
	Time	0–10	0↓**	10	20	30	40	50	60↑††	70	80	90	100	110	120	135	150	165
10†	A	75.0	76.6	69.5	59.2	53.2	48.0	46.1	45.6	54.0	67.8	67.2	71.6	80.8	79.7	82.4	85.8	83.8
	V	69.5	72.7	67.9	59.0	53.2	47.3	44.0	43.8	44.3	56.1	58.4	64.2	72.0	72.0	74.9	77.8	72.0
	A-V	5.5	3.9	1.6	0.2	0.0	0.7	2.1	1.8	9.7	11.7	8.8	7.4	8.8	7.7	7.5	8.0	11.8
	A-V/A	0.073	0.050	0.023	0.003	0.000	0.014	0.045	0.038	0.179	0.172	0.130	0.103	0.108	0.096	0.091	0.093	0.140

* Insulin infused into a foreleg vein over a two minute period.
† Insulin infused into a foreleg vein over a 60 minute period.
‡ Arterial glucose concentration.
§ Venous glucose concentration.
‖ Arteriovenous glucose difference.
¶ Arteriovenous glucose difference/arterial glucose concentration.
** ↓ Start infusion.
†† ↑ End infusion.

means for the valid measurement of A–V glucose differences as little as 2 mg. per cent (5, 7, 8).

RESULTS

The changes in femoral arterial and venous glucose concentrations, A–V glucose difference, and (A–V)/A, following the peripheral and portal administration of glucagon-free insulin are listed in Tables I and II. Typical data from two paired experiments, comparing the effects of the two routes of administration, are shown in Figures 1 and 2.

In each dog, the peripheral injection of insulin increased peripheral glucose utilization to a significantly greater extent than did the portal administration. The mean increases of 6.9 mg. per cent in A–V glucose difference and 0.097 in (A–V)/A after the injection of insulin into a foreleg vein were significantly greater ($p = < 0.01$) than the mean increases of 4.6 mg. per cent in A–V glucose difference and 0.064 in (A–V)/A after the intraportal injection of the same dose of insulin (Table III, Figures 3 and 4). Similarly, the mean peripheral utilization index of 80, computed according to the method of Somogyi (9), after insulin delivered into a peripheral vein was significantly greater ($p = < 0.01$) than the mean index of 56, following the intraportal route of administration (Table IV).

TABLE II

Changes in femoral arterial and venous glucose concentrations after glucagon-free insulin (0.07 U per Kg.) injected into a branch of the portal vein

		Control		Time after insulin administration												
Dog		0–10	0	5	10	15	20	25	30	40	50	60	75	80	90	105	120
1*	A‡	87.5	89.7		79.0		60.0		67.0	72.3	74.0	69.0	74.0		83.0	84.4	81.5
	V§	85.0	86.8		77.4		51.0		58.8	62.2	59.8	59.3	67.2		76.0	75.1	74.0
	A-V‖	2.5	2.9		1.6		9.0		8.2	10.1	14.2	9.7	6.8		7.0	9.3	7.5
	A-V/A¶	0.028	0.037		0.020		0.150		0.123	0.139	0.191	0.140	0.091		0.084	0.108	0.092
6*	A	82.7	87.0	81.4	68.0	56.4	52.8	54.8	59.2	60.2	66.0	69.7	68.2		73.7	78.0	75.0
	V	82.7	85.0	77.2	63.0	51.0	46.6	47.8	52.0	53.6	56.0	58.0	60.8		67.4	75.8	75.0
	A-V	0.0	2.0	4.2	5.0	5.4	6.2	7.0	7.2	6.6	10.0	11.7	7.4		6.3	2.2	0.0
	A-V/A	0.000	0.022	0.051	0.073	0.096	0.117	0.124	0.121	0.110	0.151	0.167	0.108		0.085	0.028	0.000
5*	A	78.5	85.6	81.7	67.4	67.5	70.0	76.0	78.0	84.8	78.8	76.1	81.0		79.1	86.7	77.7
	V	77.0	84.8	81.4	65.7	64.2	63.8	66.0	69.7	78.5	69.3	68.6	73.8		75.0	78.9	77.0
	A-V	1.5	0.8	0.3	1.7	3.3	6.2	10.0	8.3	6.3	9.5	7.5	7.2		4.1	7.8	0.7
	A-V/A	0.019	0.009	0.003	0.025	0.048	0.088	0.131	0.106	0.074	0.120	0.098	0.088		0.051	0.089	0.009
8*	A	85.2	84.2	81.8	72.8	69.2	63.2	58.8	67.4	84.7	85.7	84.2	83.6		83.6	83.6	
	V	84.2	84.2	81.8	71.4	68.0	61.7	56.4	63.8	76.3	77.8	74.4	77.0		77.8	81.0	
	A-V	1.0	0.0	0.0	1.4	1.2	1.5	2.4	3.6	8.4	7.9	9.8	6.6		5.8	2.6	
	A-V/A	0.011	0.000	0.000	0.019	0.017	0.024	0.040	0.053	0.099	0.092	0.116	0.079		0.069	0.031	
3*	A	90.6	89.4	84.6	75.6	71.2	67.2	66.7	67.6	71.0	78.1	83.4	84.6	85.1			
	V	89.4	88.3	84.0	73.0	68.8	64.6	63.4	63.0	66.7	73.8	76.0	79.1	80.2			
	A-V	1.2	1.1	0.6	2.6	2.4	2.6	3.3	4.6	4.3	4.3	7.4	5.6	4.9			
	A-V/A	0.013	0.012	0.007	0.034	0.034	0.039	0.049	0.068	0.060	0.055	0.088	0.065	0.057			
4*	A	93.6	95.0	81.8	69.0	58.0	64.3	70.3	73.8	76.8	80.8	85.8	88.4	89.4			
	V	93.2	92.7	82.0	66.0	56.0	60.2	65.3	68.1	73.1	73.8	84.0	86.6	87.6			
	A-V	0.4	2.3	−0.2	3.0	2.0	4.1	5.0	5.7	3.7	7.0	1.8	1.8	1.8			
	A-V/A	0.004	0.024	−0.002	0.043	0.034	0.064	0.071	0.077	0.048	0.086	0.020	0.020	0.020			
2*	A	100.4		105.3	91.3	86.0	78.5	75.0	72.4	80.6	86.0	91.7	96.6	98.4			
	V	99.8		102.1	90.8	85.6	78.2	74.6	70.3	78.9	84.6	90.4	96.2	98.6			
	A-V	0.6		3.2	0.5	0.4	0.3	0.4	2.1	1.7	1.4	1.3	0.4	−0.2			
	A-V/A	0.006		0.030	0.005	0.004	0.004	0.005	0.029	0.021	0.016	0.014	0.004	−0.002			
	Time	0–10	0↓**	10	20	30	40	50	60↑††	70	80	90	105	120	135	150	165
10†	A	83.0	83.6	73.0	66.5	64.9	66.0	66.5	68.4	76.2	84.6	83.0	81.4	83.8	83.0	83.1	81.8
	V	80.4	81.0	73.0	64.2	64.2	64.2	63.4	64.9	68.7	73.8	79.0	74.0	77.8	77.9	77.0	79.0
	A-V	2.6	2.6	0.0	2.3	0.7	1.8	3.1	3.5	7.5	10.8	4.0	7.4	6.0	5.1	6.1	2.8
	A-V/A	0.031	0.031	0.000	0.034	0.001	0.027	0.047	0.051	0.098	0.127	0.048	0.090	0.071	0.061	0.073	0.034

* Insulin infused into a branch of the portal vein over a two minute period.
† Insulin infused into a branch of the portal vein over a 60 minute period.
‡ Arterial glucose concentration.
§ Venous glucose concentration.
‖ Arteriovenous glucose difference.
¶ Arteriovenous glucose difference/arterial glucose concentration.
** ↓ = Start infusion.
†† ↑ = End infusion.

In contrast to the difference in peripheral glucose utilization, following the intraportal and peripheral injection of insulin, is the similarity in the magnitude of the hypoglycemia which results after both routes of insulin administration. The arterial glucose response after each route of administration, along with the standard deviation of the mean difference between these changes, are shown in Figure 5. There is no significant difference in the magnitude of the hypoglycemia between both routes of administration when calculated either in milligrams or percentage decrease from fasting levels (Table V).

In order to determine whether the changes in peripheral glucose utilization resulting from the intraportal administration of insulin were dependent upon the amount of insulin perfusing the liver per unit of time, an experiment was performed (Dog 10, Tables I and II) in which insulin (0.0017 U per Kg. per minute) was administered intraportally and intravenously over a one hour period at 1/20 the rate of the previous experiments. If the alterations in the insulin during passage through the liver were rate-limited, a larger difference between the changes after the peripheral and portal infusion of insulin would

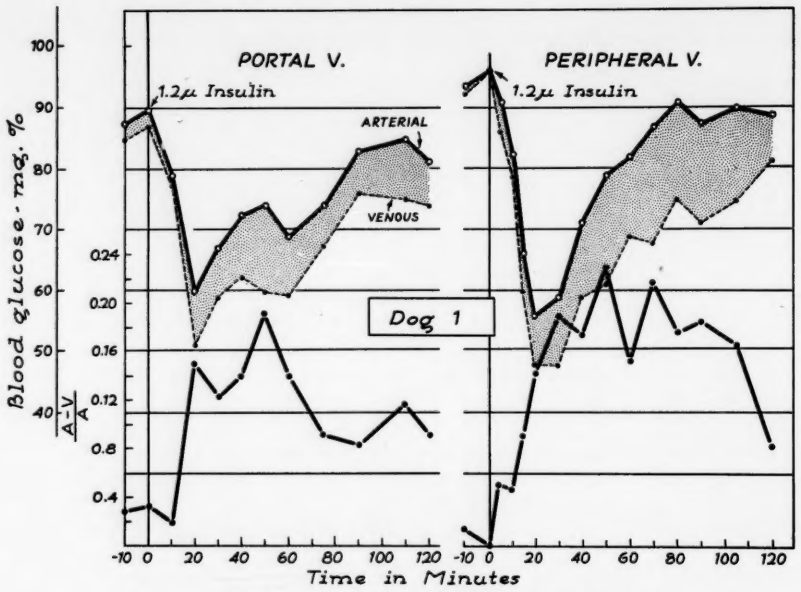

FIG. 1. ADMINISTRATION OF GLUCAGON-FREE INSULIN (DOG 1)

be forthcoming. Such was not the case; the differences in (A–V)/A and A–V glucose difference were not greater than those obtained when insulin was injected at a rate of 0.035 U per Kg. per minute. This suggests that within the range tested, this process, *in vivo*, is not rate-limited, but rather that a fixed percentage of the insulin perfusing the liver per unit time is bound. This contention

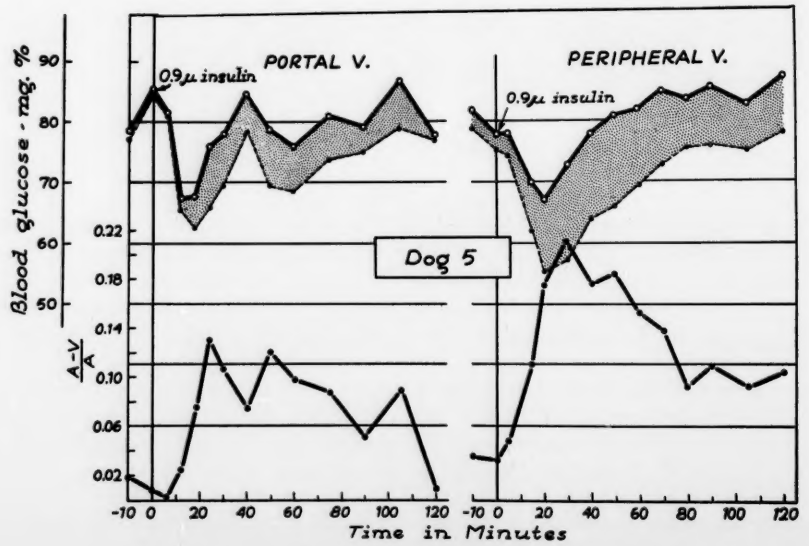

FIG. 2. ADMINISTRATION OF GLUCAGON-FREE INSULIN (DOG 5)

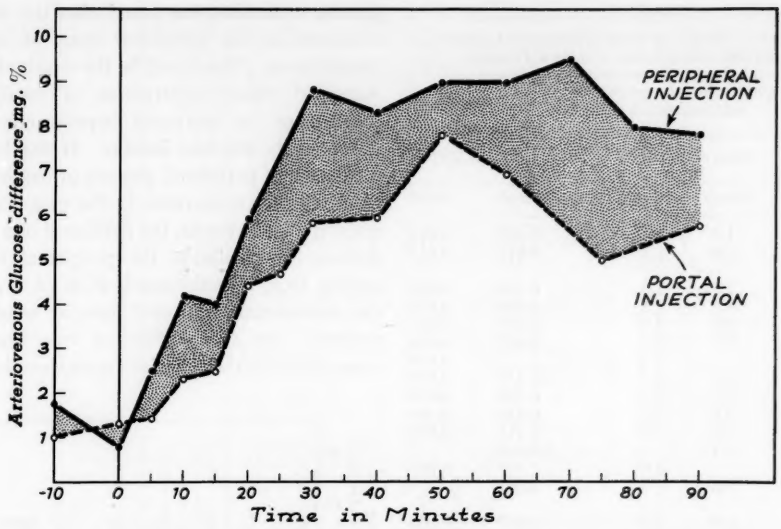

FIG. 3. COMPARISON OF CHANGES IN MEAN ARTERIOVENOUS GLUCOSE DIFFERENCE AFTER PERIPHERAL AND PORTAL INJECTION OF GLUCAGON-FREE INSULIN

is supported by the finding of Lee and Williams that an elevenfold variation in the amount of insulin injected into rats had no effect on the per cent of the dose bound in the liver within 15 minutes (10).

DISCUSSION

The direct hepatic effect of insulin has long been a controversial issue (11). While the *in vitro* effects of insulin on the liver have been particularly difficult to demonstrate, *in vivo* effects of varying magnitude and duration have been shown (12–16). The evidence supporting an hepatic effect of insulin has recently been reviewed by de Duve (12) and Dunn, Friedmann, Maass, Reichard, and Weinhouse (13). If, as this evidence indicates, insulin alters hepatic glucose metabolism, then the reduction of blood glucose concentration which follows insulin administration is the consequence, not only of an in-

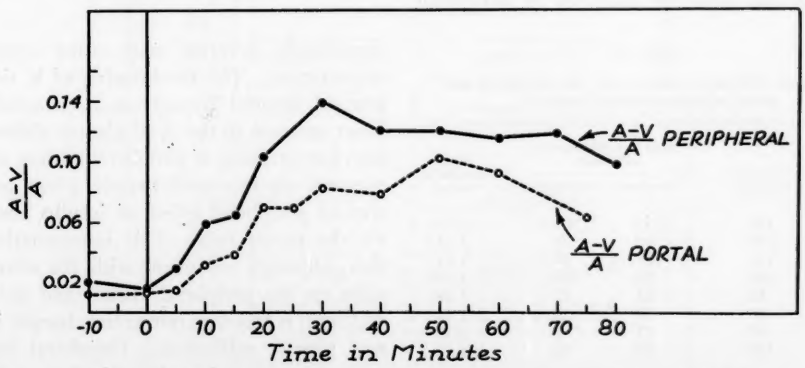

FIG. 4. COMPARISON OF MEAN CHANGES IN (A–V)*/A AFTER PERIPHERAL AND PORTAL ADMINISTRATION OF GLUCAGON-FREE INSULIN

* Arteriovenous glucose difference/arterial glucose concentration.

TABLE III

Comparison of changes in mean arteriovenous glucose difference and arteriovenous glucose difference/arterial glucose concentration with time after the intraportal and peripheral administration of insulin

Time after insulin (*minutes*)	Mean A-V* glucose (*mg.*)		Mean A-V† A	
	Periph.	Portal	Periph.	Portal
−10	1.8	1.1	0.021	0.012
0	0.8	1.3	0.016	0.014
5	2.5	1.5	0.030	0.015
10	4.2	2.2	0.059	0.031
15	3.9	2.4	0.065	0.039
20	5.9	4.3	0.103	0.069
25		4.7		0.070
30	8.8	5.7	0.139	0.082
40	8.4	5.8	0.120	0.079
50	9.0	7.7	0.120	0.102
60	8.6	7.0	0.115	0.092
70	9.4		0.118	
75		4.9		0.063
80	8.0		0.097	
Mean	6.9	4.6	0.097	0.064
p	<0.01		<0.01	

* Arteriovenous.
† Arteriovenous glucose difference/arterial glucose concentration.

creased peripheral glucose utilization, but also of a decreased hepatic output of glucose (12, 13). Increased peripheral glucose utilization in the intact animal is usually manifested by an increased A–V glucose difference (5, 9, 17); whereas the decreased hepatic output of glucose, although contributing to the hypoglycemia, does not alter the A–V glucose difference.

A significantly smaller increase in A–V glucose difference and therefore probably in peripheral glucose utilization was found after the endoportal, compared to the peripheral route of insulin administration. This could be the result of either an increased hepatic destruction of insulin by insulinase or an increased hepatic effect of the endoportally injected insulin. If the lesser augmentation of peripheral glucose utilization was accompanied by a decrease in the magnitude of the arterial hypoglycemia, the likelihood of a decreased delivery of insulin to the peripheral tissues resulting from hepatic inactivation of a portion of the intraportally injected insulin would be increased. No such difference was found. The magnitude of the arterial hypoglycemia was not

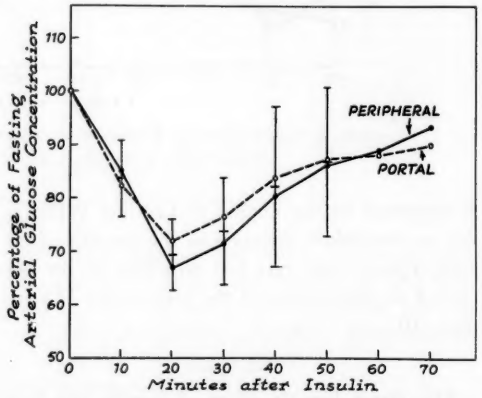

FIG. 5. COMPARISON OF MEAN ARTERIAL GLUCOSE RESPONSE AFTER THE INTRAPORTAL AND PERIPHERAL ADMINISTRATION OF GLUCAGON-FREE INSULIN (0.07 U PER KG.)

significantly different after either route of administration. The combination of a similar degree of arterial hypoglycemia, coupled with a lesser increase in the A–V glucose difference and therefore probably of peripheral glucose utilization, suggests an increased hepatic effect and a decreased peripheral effect of insulin administered via the portal vein. This interpretation of the data, although consonant with the effects of insulin on the peripheral tissues and on the liver (12, 13), is based on inferential changes in peripheral glucose utilization. Peripheral blood flow was not measured, and A–V glucose differences alone were used as the index of peripheral glucose utilization. It is therefore possible that a greater

TABLE IV

Peripheral utilization indices after the peripheral and portal administration of insulin

Dog	Duration (*minutes*)	Sum of A-V* glucose difference		Ratio peripheral/portal
		Peripheral	Portal	
1	120	153	101	1.52
6	120	90	74	1.21
5	120	129	70	1.84
8	100	73	56	1.30
3	80	55	37	1.48
4	80	38	29	1.31
2	80	17	8	2.12
10	150	86	72	1.19
Mean		80	56	1.49
p		<0.01		

* Arteriovenous.

TABLE V

Comparison of the effect on arterial glucose concentration of the intraportal and peripheral administration of insulin

Time.....	10 minutes			20 minutes			30 minutes			40 minutes			50 minutes			60 minutes		
Dog	Port.	Periph.	Diff.	Port.	Periph.	Diff.	Port.	Periph.	Diff.	Port.	Periph.	Diff.	Port.	Periph.	Diff.	Port.	Periph.	Diff.
							Milligrams decrease from fasting level after insulin											
1	10.7	13.6	+ 2.9	29.7	40.0	+10.3	22.7	37.0	+14.3	17.4	24.7	+ 7.3	15.7	17.0	+ 1.3	20.7	14.0	− 6.7
6	19.0	13.6	− 5.4	34.2	27.2	− 7.0	27.8	34.7	+ 6.9	26.8	21.6	− 5.2	21.0	13.2	− 7.8	17.3	13.2	− 4.1
5	18.2	4.0	−16.2	15.6	10.8	− 4.8	7.6	5.2	− 2.4	0.8	0.0	− 0.8	6.8	−3.0	− 9.8	9.5	−4.0	−13.5
8	11.4	14.1	+ 2.7	21.0	31.3	+10.3	16.8	25.9	+ 9.1	−0.5	13.1	+13.6	−1.5	8.3	+ 9.8	0.0	5.4	+ 5.4
3	13.8	12.7	− 1.1	22.2	25.6	+ 3.4	21.8	11.8	−10.0	18.4	4.0	−14.4	11.3	2.7	− 8.6	6.0	−0.2	− 6.2
4	26.0	21.3	− 4.7	30.7	36.9	+ 6.2	21.2	16.1	− 5.1	18.2	12.3	− 5.9	14.2	5.5	− 8.7	9.2	6.7	− 2.5
2	12.1	8.3	− 3.8	24.9	27.1	+ 2.2	31.0	41.0	+10.0	22.8	43.5	+20.7	17.4	39.9	+22.5	11.7	32.9	+21.2
10	10.6	7.1	− 3.5	17.1	17.4	+ 0.3	18.7	23.4	+ 4.7	17.6	28.6	+11.0	17.1	30.5	+13.4	15.2	31.0	+15.8
Mean	15.2	11.8	− 3.4	24.4	27.0	+ 2.6	21.0	24.5	+ 3.5	15.2	18.5	+ 3.3	12.8	14.3	+ 1.5	11.2	12.5	+ 1.3
	p = >0.1			p = >0.4			p = >0.2			p = >0.4			p = >0.5			p = >0.5		
							Per cent decrease from fasting after insulin											
1	11.9	14.2	+ 2.3	33.1	41.7	+ 8.6	25.3	38.5	+13.2	19.4	25.7	+ 6.3	17.5	17.7	+ 0.2	23.1	14.6	− 8.5
6	21.8	18.2	− 3.6	39.3	36.5	− 2.8	32.0	46.5	+14.5	30.8	29.0	− 1.8	24.1	17.7	− 6.4	19.9	17.7	− 2.2
5	21.3	5.1	−16.2	18.2	13.8	− 4.4	8.9	6.7	− 2.2	0.9		− 0.9	7.9	−3.8	−11.7	11.1	−5.1	−16.2
8	13.5	16.0	+ 2.5	24.9	35.5	+10.6	20.0	29.4	+ 9.4	−0.6	14.9	+15.5	−1.8	9.4	+11.2	0.0	6.1	+ 6.1
3	15.4	15.9	+ 0.4	24.8	32.0	+ 7.2	24.4	14.8	− 9.6	20.6	5.0	−15.6	12.6	3.4	− 9.2	6.7	−0.3	− 7.0
4	27.4	24.1	− 3.3	32.3	41.8	+ 9.5	22.3	18.2	− 4.1	19.2	13.9	− 5.3	14.9	6.2	− 8.7	9.7	7.6	− 2.1
2	11.7	9.1	− 2.6	24.1	29.9	+ 5.8	30.0	45.2	+15.2	22.0	48.0	+26.0	16.8	44.0	+27.2	11.3	36.3	+25.0
10	12.6	9.1	− 3.5	20.4	22.4	+ 2.0	22.3	30.1	+ 7.8	21.0	36.8	+15.8	20.4	39.3	+18.9	18.1	20.4	+ 2.3
Mean	17.0	14.0	− 3.0	27.1	31.7	+ 4.6	23.2	28.7	+ 5.5	16.7	21.7	+ 5.0	14.1	16.7	+ 3.6	12.5	12.2	− 0.3
	p = >0.1			p = >0.05			p = >0.1			p = >0.3			p = >0.5			p = >0.5		

hypoglycemic effect from either route of administration was masked by the counterregulatory reactions which not only raised blood glucose concentrations, but also may have altered peripheral blood flow.

The difference between the metabolic effects of insulin injected endoportally and peripherally is probably causally linked with the increased amount of insulin, which reaches the liver after endoportal administration. Although the time of exposure of the liver to this larger amount of insulin is only a few seconds, *i.e.*, the duration of a single circulation through the liver, other data indicate that exposure of an extremity to a large amount of insulin for a similar duration of time also augments the metabolic response of that limb (17). Bell and Burns showed that the intra-arterial injection of insulin resulted in a significantly greater A–V glucose difference in the injected leg compared to the contralateral extremity (17). In the intact animal, after the intravenous injection of insulin, the magnitude of the metabolic response of a tissue sensitive to insulin would be expected to depend, not only upon the affinity of the tissue for insulin, but also upon the per cent of the cardiac output perfusing the tissue per unit time, *i.e.*, on the delivery of insulin to the tissue. When endogenous insulin is secreted into the portal vein, or exogenous insulin is injected endoportally, *all* of the insulin reaches the liver; by contrast, when insulin is injected into a peripheral vein, only a fraction of the amount given perfuses the liver.

Previous studies comparing the endoportal and peripheral routes of insulin administration were different from the present study. Arteriovenous glucose differences were not measured, and the studies were concerned primarily with the hyperglycemic effect of insulin (18, 19) presumably contaminated with glucagon. Weisberg, Freidman, and Levine (20) compared the hypoglycemic effects of the endoportal and peripheral routes of insulin administration, but they too did not measure A–V glucose differences. In contrast to the results of the present study, Weisberg, Freidman, and Levine found a greater hypoglycemic effect after the peripheral route of injection. This was attributed to the hepatic inactivation or destruction (21–23) of insulin following portal injection. However, in all but two of their experiments insulin known to contain glucagon was used. The use of glucagon-containing insulin may have obscured any differences in the metabolic effects of insulin given endoportally and peripherally for at least two reasons: the hepatic glycogenolytic effect of the glucagon may have counterbalanced or masked the hepatic effect of insulin; glucagon may have augmented peripheral glucose utilization (6, 24). For these reasons the effects obtained with insulin known to contain glucagon are not comparable to those obtained with glucagon-free insulin.

The data suggest that the secretion of insulin into the portal vein has physiologic significance. By traversing the liver first, a greater hepatic effect and a smaller peripheral effect of insulin appear to be forthcoming, compared to that which follows the peripheral administration. This physiologic balance between the magnitude of the hepatic effect and the magnitude of the peripheral effect is apparently not duplicated by the peripheral administration of insulin. This finding may be of particular importance in experiments designed to demonstrate an hepatic effect of insulin and in the study of possible betacytotropic agents such as tolbutamide which may stimulate the endogenous secretion of insulin (25). The disparate views concerning the hepatic effect of insulin (11–13) and also the comparative effects of tolbutamide and insulin on peripheral utilization and glucose intermediaries (26–28) may have their explanations in the data herein presented.

SUMMARY AND CONCLUSIONS

Sixteen experiments were performed on eight dogs. In each dog during one experiment, glucagon-free insulin was administered via the portal vein to simulate the route traversed by endogenously secreted insulin and during the other, the same dose was administered into a peripheral vein in the foreleg. The endoportal administration of insulin resulted in the same degree of arterial hypoglycemia as the peripheral route of administration, and in a significantly reduced augmentation of peripheral glucose utilization, as evidenced by changes in the A–V glucose difference. These data suggest that a physiologic difference in the metabolic pattern, after insulin administration, occurs depending upon the route of administration. Endogenous insulin secreted into the portal

vein may eventuate in a physiologic balance between the magnitude of the hepatic effect and the magnitude of the peripheral effect, which is apparently not duplicated by the peripheral administration of insulin.

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OBSERVATIONS ON CARBON DIOXIDE TENSION DURING RECOVERY FROM METABOLIC ACIDOSIS [1]

By ROBERT W. WINTERS,[2] JOHN A. LOWDER,[3] AND NELSON K. ORDWAY [4]

(From the Departments of Pediatrics and Medicine, University of North Carolina School of Medicine, Chapel Hill, N. C.)

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The importance of hyperventilation in metabolic acidosis in minimizing the shift in blood pH is well known. It has been generally assumed that the stimulus for this augmented ventilation is the increased hydrogen ion concentration of the blood. If such were the case, it might be further assumed that during recovery from metabolic acidosis, the ventilation and hence the carbon dioxide tension would be adjusted in accordance with the pH of the blood.

This report presents data bearing upon the recovery of acid-base equilibrium in patients with metabolic acidosis due to diabetes, to diarrheal disease and to renal disease. The results show the frequent occurrence of normal or even alkaline blood pH values associated with reduced pCO_2 values during the recovery phase, and imply that some degree of hyperventilation persists in the absence of a measurable chemical stimulus in the blood.

METHODS

The data presented include measurements of whole blood pH, plasma carbon dioxide content, and calculated plasma carbon dioxide tension (pCO_2) in 10 children and 1 adult with diabetic acidosis, in 27 infants and children with diarrheal acidosis, and in 3 children and 5 adults with uremic acidosis who were studied on the wards of the North Carolina Memorial Hospital, Chapel Hill, and the L.S.U. division of Charity Hospital, New Orleans. In nearly every instance, heparinized venous blood drawn without stasis was used; the remaining few determinations were performed on arterial blood.

Whole blood pH was determined by the glass electrode, either at 37° C., or at room temperature and corrected to 37° C. using Rosenthal's (1) temperature coefficient. Total carbon dioxide content was determined by the method of Van Slyke and Neill (2). Plasma carbon dioxide tension (pCO_2) was calculated according to the Henderson-Hasselbalch equation, using a value of 6.11 for pK′ and a CO_2 solubility factor of 0.0311 (3).

RESULTS

Diabetic acidosis

Figure 1 shows the acid-base data for the diabetic patients, together with similar data from various sources in the literature (4–11). In this and in subsequent figures, the total carbon dioxide content of the plasma is plotted logarithmically on the ordinate, while whole blood pH is plotted on the abscissa. On such a plot, the loci for various values of carbon dioxide tension for arterial blood at 37° C. are defined by the nearly straight lines which transect the major axes. Two points are plotted for each patient, the first representing the data obtained prior to treatment, and the second representing the earliest observation of a normal or alkaline pH after institution of treatment. The latter point was obtained in two hours to four days; in 39 of 51 patients this observation was within 24 hours.

Prior to treatment, all patients were in metabolic acidosis, the average pH for the entire group being 7.08, and the average total carbon dioxide content being 7.4 mM per L. All patients received insulin and parenteral fluids, and in some instances (literature cases only), large amounts of either sodium bicarbonate or sodium lactate.

The post-treatment data show that most of the patients attained blood pH values of 7.35 or greater, while the pCO_2 was still low. This is unequivocally the case in 39 of the 51 patients. These findings are especially significant in view of the fact that over two-thirds of the data of Figure 1 was obtained from venous blood; had arterial data been available, the pCO_2 values would

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[1] Presented at the Eleventh Annual Meeting of the Southern Society for Clinical Research, New Orleans, January 26, 1957.

[2] Postdoctoral Fellow, National Foundation for Infantile Paralysis.

[3] Present address: Department of Medicine, University of Alabama School of Medicine, Birmingham, Ala.

[4] Present address: Department of Pediatrics, Yale University School of Medicine, New Haven, Conn.

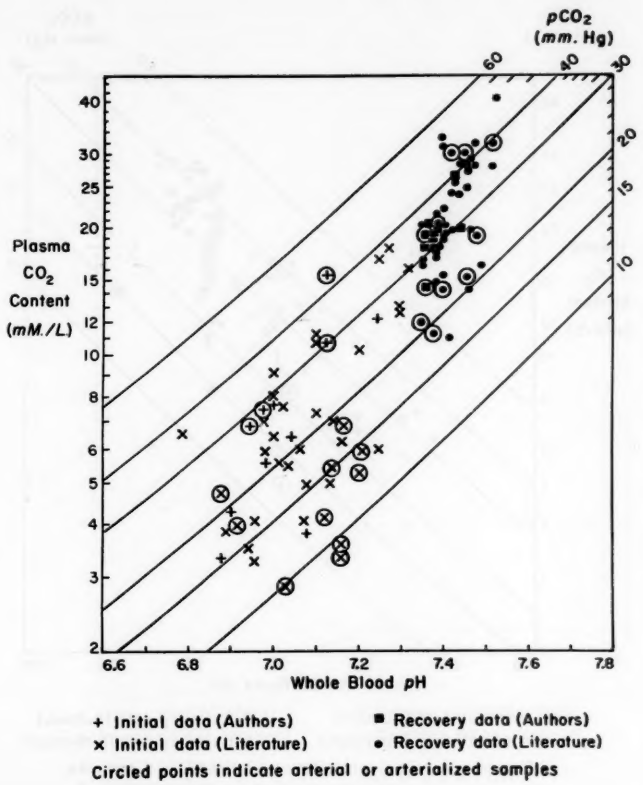

FIG. 1. DIABETIC ACIDOSIS

have been even lower than those shown in the figure.

Diarrheal acidosis

Data similar to those of the diabetic patients are shown in Figure 2 for 27 infants and children with diarrhea, along with similar data from the literature (12). The initial data show that all patients were in a state of metabolic acidosis (average pH, 7.22; average CO_2 content, 13.3 mM per L.). Although the post-treatment point on each patient was, in general, reached at a longer interval after the first than in the diabetic subjects (24 of the 42 within 2 days; range, 6 hours to 17 days), the same phenomenon is apparent in these cases also in that 30 of the 42 patients achieved a normal or supernormal pH associated with a pCO_2 of less than 40 mm. Hg. Here, too, the use of acid-base data from venous blood masks the even greater depression of pCO_2 present in the arterial blood of the patients.

Uremic acidosis

In Figure 3 are shown data derived from a study of eight uremic patients before and from 14 hours to 4 days after therapy consisting of rehydration, which in most instances included sodium bicarbonate infusions. Also shown are data of Yeomans and Stueck (13) on four patients managed in a simliar manner, as well as data from the report of Weller, Swan, and Merrill (14) on uremic patients before and from four to six hours after extracorporeal dialysis. As in the case of the patients with diabetic and diarrheal acidosis, most of the patients with uremic acidosis attained a

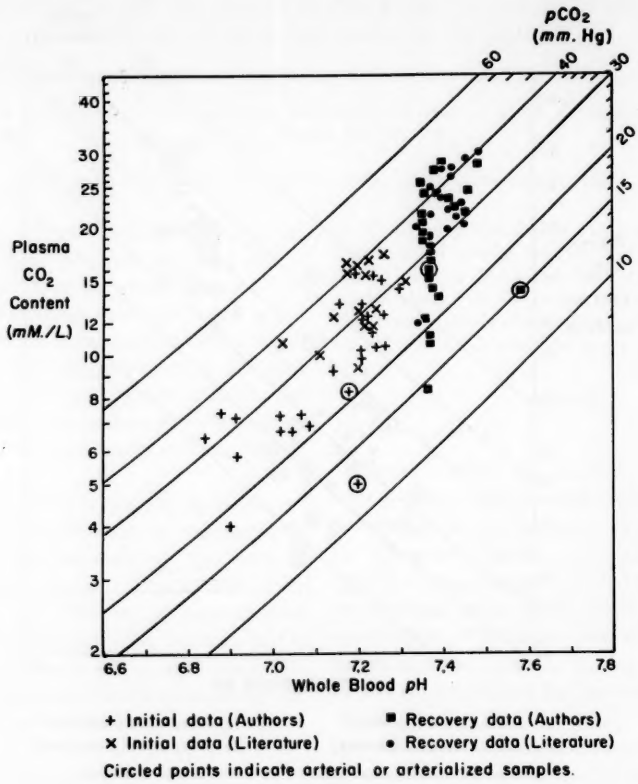

FIG. 2. DIARRHEAL ACIDOSIS

normal or alkaline pH associated with distinctly depressed values for pCO_2.

DISCUSSION

The data presented clearly show that a majority of the patients recovering from diabetic acidosis pass through a point at which the pH is normal or elevated but the pCO_2 is depressed. This observation, apparently first made by Peters (15) in 1917, has been repeatedly confirmed (4–11, 16) in diabetic patients.

Delayed return of pCO_2 to normal appears to be a feature not unique to diabetic acidosis, since a similar phenomenon was observed in patients recovering from diarrheal acidosis and uremic acidosis. Furthermore, published observations by others (17, 18) on subjects recovering from ammonium chloride acidosis show a similar pattern (see Figure 3).

The findings of pCO_2 values lower than normal suggest that hyperventilation is in some measure sustained, a contention supported by ventilation measurements reported by Kety, Polis, Nadler, and Schmidt (8). The mechanisms underlying the continued hyperventilation by such patients are not clear. It is apparent that within the confines of Gray's multiple factor theory for the chemical control of pulmonary ventilation (19) there is no discernible stimulus in the arterial blood for the augmented ventilation, since pCO_2 is reduced, hydrogen ion is either normal or reduced, and pO_2 is presumably elevated due to the hyperventilation.

One explanation might be that the sensitivity of the respiratory center to pCO_2 and/or hydrogen

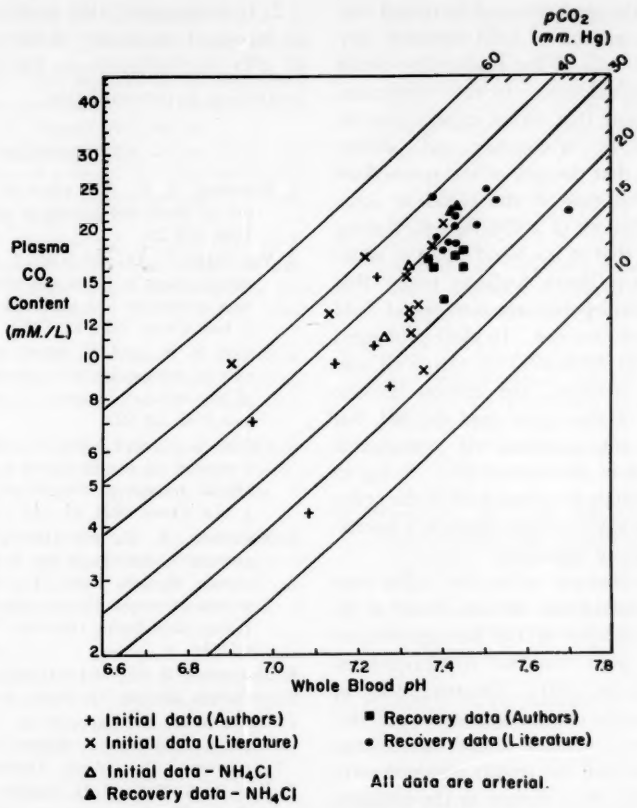

FIG. 3. UREMIC AND NH_4Cl ACIDOSIS

ion has been increased. Increased sensitivity, assessed by a change in the slope of the curve relating the alveolar ventilation ratio to arterial pCO_2 during inhalation of various concentrations of carbon dioxide, has indeed been demonstrated in other situations in which there has been sustained *hypo*capnia but with alkalosis; *viz.*, passive hyperventilation of normal subjects (20) and acclimatization to high altitude (21). The opposite response, decreased sensitivity to pCO_2, has been adequately demonstrated in chronic *hyper*capnic states (22–25).

Direct studies dealing with the sensitivity to pCO_2 of subjects with metabolic acidosis have been reported in only a few instances. Tenney (22) showed a clearcut increase in one patient with a chronic ammonium chloride acidosis and a reversion to normal after full recovery from the acidosis. Alexander, West, Wood, and Richards (23) found increased sensitivity in one of three patients with mild degree of metabolic acidosis secondary to chronic renal disease, while Nielson (26) detected only slight or no change with acute ammonium chloride acidosis. These conflicting results obtained by different investigators may be due to the different intensities and different durations of the acidosis present in the subjects and are not necesarily incompatible with the explanation offered for the findings reported above.

The mechanisms mediating increased sensitivity of the respiratory center are not understood. A reduction in the buffer capacity of the blood is hardly a single satisfactory explanation, since this would not explain the development of the frankly alkalotic pH values which were observed in some of the patients.

It is likely that the mechanism of increased sensitivity involves alteration of local chemical conditions, *i.e.*, in the cells of the respiratory center or in the cerebrospinal fluid. In this connection, it is pertinent to note that recent experiments by Leusen (27) and by Winterstein and Gökhan (28) have shown that the pH of the spinal fluid varies directly with that of the blood in acute respiratory disturbances of acid-base equilibrium, but inversely with that of the blood in acute metabolic disturbances. These findings imply that carbon dioxide freely permeates into spinal fluid but that bicarbonate does not. In more prolonged experiments (up to 48 hours) in cats with ammonium chloride acidosis, the carbon dioxide combining power of the spinal fluid did fall, but the degree was not quantitatively comparable with that observed in the blood (29). A lag in recovery of bicarbonate in spinal fluid of the order suggested by these studies might provide a mechanism for the change in sensitivity.

With respect to changes within the buffer content of the cells themselves, Brown found a reduced buffering capacity of the homogenates of brains of guinea pigs who had hyperventilated secondary to hypoxia (30). The magnitude of the change was such as to suggest that a real change in the buffer content of the intracellular fluids had occurred and the results obtained were not explicable solely by a change in the extracellular phase of the tissue.

In terms of the concept of alteration of local chemical environment, the findings observed in subjects recovering from metabolic acidosis could be explained if there was a significant lag in the recovery of the buffer capacity of the cerebrospinal fluid or the cells themselves, whereby respiration and hence carbon dioxide tension were controlled primarily by local conditions rather than by the pH and pCO_2 of the arterial blood.

SUMMARY

1. Patients recovering from diabetic, diarrheal or uremic acidosis frequently pass through a phase at which blood pH is normal or supernormal but at which pCO_2 is still depressed. This suggests that there is sustained hyperventilation on the part of such patients even though they are no longer acidotic.

2. It is suggested that such patients may have an increased sensitivity of the respiratory center to pCO_2 and/or hydrogen ion to account for the continuing hyperventilation.

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STUDIES OF THE MECHANISM OF THE EFFECT OF HOG INTRINSIC FACTOR CONCENTRATE ON THE UPTAKE OF VITAMIN B_{12} BY RAT LIVER SLICES [1]

By VICTOR HERBERT [2]

(From the Department of Medicine, Albert Einstein College of Medicine, Bronx Municipal Hospital Center, New York, N. Y.)

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An enhancement of the uptake of Co^{60}-labeled vitamin B_{12} by rat liver slices in the presence of hog intrinsic factor concentrate (HIFC) was reported by Miller, Raney, and Hunter (1), and has been confirmed by Latner and Raine (2). This report is concerned with the mechanism of this enhancement. A preliminary report of this work has been presented (3).

MATERIALS AND METHODS

The basic protocol for each experiment was the following: a healthy adult male rat of the Sherman strain was sacrificed. His liver was immediately removed and placed in 0.9 per cent NaCl at 0° C. The liver was sliced with a Stadie-Riggs microtome designed to prepare slices 0.5 mm. thick. Each slice was immediately placed in a 20 ml. beaker containing 5 ml. of incubation medium and kept in a mixture of water and crushed ice at 0° C. The standard incubation medium was Hastings' bicarbonate buffered at pH 7.8 (4). The beaker and medium were weighed before and after addition of the slices, and the weight of the slices was calculated by difference. The weight of liver slices used was 200 to 300 mg. per incubation vessel.

Incubation medium was then added to each beaker to a final volume of medium in milliliters equal to 30 times the weight of the slices in grams (*i.e.*, when the slices were calculated to weigh 0.2 Gm., the volume of the medium was made up to 6 ml.).

A preparation of HIFC [3] was next added, contained in a volume of 0.9 per cent NaCl equal to 10 per cent of the final volume of the incubation medium (*i.e.*, when the final volume of the medium was 6 ml., the HIFC was added in 0.6 ml. of 0.9 per cent NaCl). Vitamin B_{12}-Co^{60} [4] (specific activity 1 μc. per μg.) was then added, also in a volume of 0.9 per cent NaCl equal to 10 per cent of the final volume of the incubation medium. Equivalent volumes of 0.9 per cent NaCl, without HIFC, but with vitamin B_{12}-Co^{60}, were added to control samples. The final concentration of vitamin B_{12}-Co^{60} was 830 $\mu\mu$g. (830×10^{-12} Gm.) and that of HIFC was 0.27 μg. per ml. This concentration of vitamin B_{12}-Co^{60} was chosen as a convenient compromise between the upper limits of the physiologic level of vitamin B_{12} in rat serum and the lower limits of adequate vitamin B_{12}-Co^{60} for accurate scintillation counting. At this concentration of vitamin B_{12}-Co^{60}, adsorption onto glass was very slight.

Incubation was performed in a Dubnoff metabolic shaker-incubator. The gas phase was 95 per cent O_2-5 per cent CO_2, shaking was at 92 cycles per minute, and the period of incubation was three hours.

At the termination of incubation, the supernatant solution was decanted and the slices were washed in 30 volumes of incubation medium at 3° C. After washing, the slices were incubated for five minutes in another 30 volumes of incubation medium at 37.5° C., the medium was decanted, and a final wash in 30 volumes of incubation medium at 3° C. was performed.

TABLE I

Uptake of vitamin B_{12}-Co^{60} by rat liver slices in three incubation media—hog intrinsic factor concentrate

Incubation medium		Counts per minute per gram of slices
Experiment A:		
Standard	Control	280
	HIFC*	2,020
Experiment B:		
Standard, with pyruvate added	Control	140
	HIFC	1,330
Krebs-Ringer phosphate	Control	220
	HIFC	750
	HIFC × 4.3	860
	HIFC × 0.05	220

* Hog intrinsic factor concentrate.

[1] Supported in part by a grant (H-2803) from the National Institutes of Health of the United States Public Health Service and in part by a grant from Lederle Laboratories, Pearl River, New York.

[2] Senior Research Fellow, New York Heart Association and Heart Fund, Inc.

[3] No. WES655, kindly provided by Dr. W. L. Williams and Dr. L. Ellenbogen, Lederle Laboratories, Pearl River, New York. This preparation was clinically active in a daily oral dose of 1 mg., as determined by urinary excretion studies of the Schilling type (5).

[4] Purchased from Merck, Sharpe & Dohme, Rahway, New Jersey, as a stock solution containing 1 μg. vitamin B_{12}-Co^{60} per ml., with a specific activity of 1 μc. per μg.

Radioactivity retained in the slices was determined in a well-type scintillation counter. Each sample was counted for a minimum of 10,000 counts. Radioactivity was expressed as counts per gram of wet liver slices per minute.

All experiments were done in duplicate. In each table, the counts of duplicate samples have been averaged and the average reported. Individual samples rarely exceeded a 5 per cent deviation from the average, except where total counts were less than 300.

RESULTS

Buffers

In standard medium, with or without added pyruvate (4), the uptake of vitamin B_{12}-Co^{60} by liver slices incubated with HIFC is as much as tenfold that observed in controls (Table I).

In Krebs-Ringer phosphate buffer at pH 7.2 (6), addition of HIFC resulted in uptakes of vitamin B_{12}-Co^{60} that were two to three times greater than controls (Table I). The effect of HIFC is not manifested at a 1 : 20 dilution and is increased only slightly, if at all, by a 4.3-fold concentration of HIFC.

The importance of buffer *per se* is open to question, since 0.9 per cent NaCl containing $CaCl_2$ was as good as the best buffer studied (Table II).

TABLE II

Effect of altering the ionic composition of the incubation medium on vitamin B_{12}-Co^{60} uptake of rat liver slices in the presence or absence of added hog intrinsic factor concentrate

Incubation medium		Counts per minute per gram of slices
Experiment F:		
Standard	Control	370
	HIFC*	2,620
Standard with bicarbonate replaced by chloride	Control	420
	HIFC	1,410
Standard with magnesium replaced by potassium	Control	440
	HIFC	2,640
Standard with calcium replaced by potassium	Control	390
	HIFC	40
Experiment G:		
Standard	Control	340
	HIFC	1,160
Standard with potassium replaced by sodium	Control	350
	HIFC	1,480
0.9% NaCl	Control	810
	HIFC	150
0.9% NaCl containing 10 mM $CaCl_2$	Control	400
	HIFC	1,260

* Hog intrinsic factor concentrate.

TABLE III

Effect of heating or 2,4-dinitrophenol on ability of rat liver slices to take up vitamin B_{12}-Co^{60} in the presence or absence of active or heat inactivated hog intrinsic factor concentrate

		Counts per minute per gram of slices
Experiment C:		
Standard experimental conditions	Control	250
	HIFC*	2,090
Slices preheated	Control	190
	HIFC	190
2,4-dinitrophenol added	Control	230
	HIFC	2,060
Experiment D:		
Standard experimental conditions	Control	560
	HIFC	1,790
	HIFC heated 10 min.	650
Experiment E:		
Incubation for 1 hour	Control	420
	HIFC	930
	HIFC heated 15 min.	430

Heating liver slices

Immersion of the beaker containing liver slices in a boiling water bath for 10 minutes prior to incubation had no measurable effect on the retained radioactivity of control liver slices (Table III). However, such heating abolished the enhancement of vitamin B_{12}-Co^{60} uptake associated with the addition of HIFC.

Heating HIFC

The addition of HIFC which had been heated in a water bath at 100° C. resulted in slight, if any, enhancement of uptake of vitamin B_{12}-C^{60} (Table III). The differences in increase of uptake with unheated HIFC in experiments C and D are due to variation from rat to rat.

2,4-dinitrophenol

Addition of 2,4-dinitrophenol in a concentration of 10^{-4}M did not diminish the influence of HIFC on the uptake of vitamin B_{12}-Co^{60} (Table III).

Time of incubation

In the presence of HIFC, the rise in vitamin B_{12}-Co^{60} uptake throughout the first hour appears

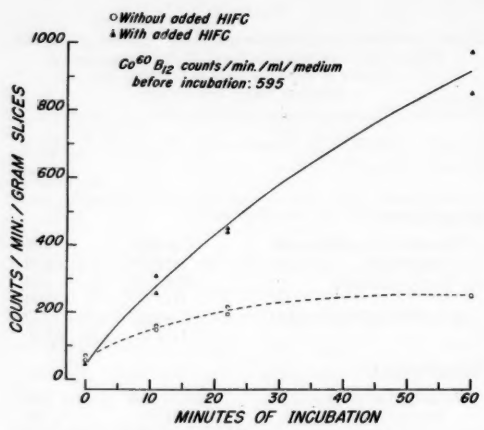

FIG. 1. EFFECT OF TIME OF INCUBATION ON UPTAKE

to be quite rapid, with decrease in uptake per unit time appearing subsequently (Figures 1 and 2).

Temperature

It is noteworthy that the effect of HIFC in enhancing the uptake of vitamin B_{12}-Co^{60} is demonstrable at 3° C. as well as at 37.5° C. (Figure 2).

Ionic composition of incubation medium

Each of the ions of the standard medium, with the exception of chloride, was replaced in turn with an equivalent millimolar concentration of another ion present in the incubation medium (Table II).

No significant effect on vitamin B_{12}-Co^{60} uptake was observed in control slices regardless of the ion replaced. The enhancing effect of HIFC was demonstrable in the absence of any one ion *except calcium*. In the absence of calcium, HIFC actually decreases the uptake of vitamin B_{12}-Co^{60}. Unpublished observations indicate the explanation of this phenomenon is that the vitamin B_{12}-Co^{60} is all bound to HIFC, and therefore unavailable to the liver slices except in the presence of calcium.

Incubation of liver slices in 0.9 per cent NaCl with and without added calcium results in uptakes of vitamin B_{12}-Co^{60} similar to those observed in the standard medium with and without calcium (Table II).

The uptake of vitamin B_{12}-Co^{60} in the presence of added HIFC was greater at a concentration of 10 mM calcium than at 1 mM. At 0.1 mM, there was no enhancing effect on addition of HIFC (Table IV).

The possibility was investigated that a divalent ion close to calcium in the periodic table might substitute for calcium. Strontium was found to be an effective substitute; other ions studied were not. These included: magnesium, cobalt, barium, cadmuim, mercury, tin, zinc, and beryllium. For these studies Krebs-Ringer solution buffered at pH 7.5 with 0.1 M trishydroxy-amino-methane buffer (7) was used. In this buffer, 10 mM concentrations of all the ions tested could be reached, (except in the cases of barium, tin, and zinc, where somewhat lower concentrations had to be used).

Effect of ethylenediaminetetraacetate (EDTA)

Liver slices were incubated for one hour and then washed in the standard manner. Aliquots were incubated for a second hour in 20 volumes of standard buffer, to which was added 2 volumes of 2 Gm. per cent disodium ethylenediaminetetraacetate dihydrate (EDTA) or 2 volumes of 0.9 per cent NaCl.

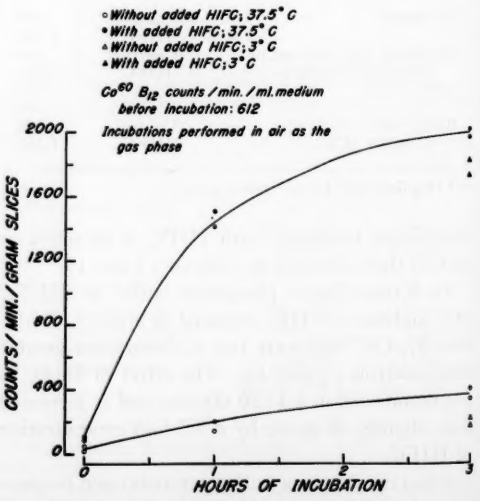

FIG. 2. EFFECT OF TIME AND TEMPERATURE OF INCUBATION ON UPTAKE

As noted in Table V, the addition of EDTA did not bring about any greater loss of vitamin B_{12}-Co^{60} from controls than did the addition of 0.9 per cent NaCl. However, the addition of EDTA markedly reduced the amount of vitamin B_{12}-Co^{60} retained in liver slices previously incubated in the presence of added HIFC.

Heparin, chondroitin sulfate, and orosomucoid

No increase in vitamin B_{12}-Co^{60} uptake by rat liver slices was demonstrated on addition to the incubation medium of heparin, chondroitin sulfate,[5] or orosomucoid,[6] in concentrations of 0.26 or of 8.3 μg. per ml. of incubation medium.

DISCUSSION

The studies here reported demonstrate certain aspects of the enhancement of the uptake of vitamin B_{12}-Co^{60} by rat liver slices in the presence of HIFC. The enhancement occurs in the cold as well as at 37.5° C. It is observable only in the presence of calcium (or strontium) in adequate concentration. It does not occur if either the liver slices or the HIFC are heated prior to incubation. The presence of 2,4-dinitrophenol in 10^{-4} M concentration does not diminish the effect of HIFC. The major portion of the vitamin B_{12}-Co^{60} taken up by rat liver slices in the presence of HIFC may be subsequently removed by incubation in the presence of EDTA.

TABLE IV

Effect of varying the calcium concentration of the incubation medium on the vitamin B_{12}-Co^{60} uptake of rat liver slices in the presence or absence of added hog intrinsic factor concentrate

Calcium concentration (mM)		Counts per minute per gram of slices
10.0	Control	860
	HIFC*	3,170
1.0	Control	680
	HIFC	1,880
0.1	Control	630
	HIFC	570
0.01	Control	630
	HIFC	470

* Hog intrinsic factor concentrate.

[5] Kindly provided by Dr. D. Hamerman.

[6] Kindly provided by Dr. G. B. J. Glass, from a lot prepared by Dr. R. J. Winzler.

TABLE V

Effect of ethylenediaminetetraacetate on retention by rat liver slices of vitamin B_{12}-Co^{60} taken up previously in the presence or absence of added hog intrinsic factor concentrate

Second incubation		Counts per minute per gram of slices
Not performed	Control	490
	HIFC*	1,210
Performed, no EDTA† added	Control	290
	HIFC	900
Performed, EDTA added	Control	290
	HIFC	360

* Hog intrinsic factor concentrate.
† Ethylenediaminetetraacetate

These data suggest that the mechanism whereby HIFC enhances vitamin B_{12}-Co^{60} uptake by rat liver slices is physical rather than metabolic. Should this interpretation be correct, the possibility would remain that vitamin B_{12}-Co^{60} held to the liver slice by HIFC might subsequently be acted on by metabolic processes within the cell.

Heparin, chondroitin sulfate, and orosomucoid do not increase vitamin B_{12}-Co^{60} uptake by rat liver slices. This would indicate that enhancement of vitamin B_{12} uptake is not a nonspecific property of any mucopolysaccharide or mucoprotein.

SUMMARY

Vitamin B_{12} uptake by rat liver slices is enhanced markedly in the presence of hog intrinsic factor concentrate. This enhancement is observable at 37.5° C., at 3° C., and in the presence of 10^{-4}M 2,4-dinitrophenol. It requires calcium (or strontium), and is reversible to an appreciable extent by EDTA.

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STUDIES OF RIBOSE METABOLISM. III. THE PATHWAY OF RIBOSE CARBON CONVERSION TO GLUCOSE IN MAN[1]

By HOWARD H. HIATT

(From the Departments of Medicine, Harvard Medical School and Beth Israel Hospital, Boston, Mass.)

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The conversion of ribose carbon to hexose via the reactions of the pentose phosphate pathway has been observed in studies with rat liver homogenates (1), rat liver slices (2), and in the intact mouse (3). Since most of these reactions are reversible (4), they provide mechanisms not only for ribose conversion to hexose, but for ribose synthesis as well. Indeed, recent work has demonstrated that a human carcinoma cell in tissue culture (5) as well as the intact human subject (6) synthesize ribose via this pathway. This report provides further evidence that in man the reactions of this pathway mediate the interconversion of ribose and glucose. D-Ribose specifically labeled with radioactive carbon was given to a patient with inoperable carcinoma. Isotope distribution in glucose subsequently isolated from this patient's blood is consistent with the conversion of ribose carbon to hexose via the pentose phosphate pathway.

METHODS

Sixty microcuries of D-ribose-1-C^{14} (24 mg.) in 10 ml. of isotonic saline was sterilized by passage through a sintered glass bacteriological filter. This solution was administered intravenously to a 75 year old female three hours after breakfast. The patient was suffering from carcinoma of the head of the pancreas, demonstrated to be inoperable on abdominal laparotomy, but her fasting blood sugar level, renal function, and hepatic parenchymal function (serum thymol turbidity, cephalin flocculation, albumin and globulin, and cholesterol, total and esterified) were normal. Fifteen minutes and two hours following the ribose injection, 50 ml. of heparinized blood was removed from an antecubital vein of the opposite arm. The blood was treated with 3 ml. of 70 per cent perchloric acid, and the protein precipitate removed by centrifugation and washed with 10 ml. of water. The supernatant solution and wash were brought to pH 7.0 with 5.0 N KOH, and the insoluble potassium perchlorate removed by centrifugation. The supernatant solution was treated with barium hydroxide and zinc sulfate (7) to remove a small amount of residual protein. Following centrifugation the clear supernatant solution was reduced to a volume of approximately 30 ml. by lyophilization and then deionized by passage through a mixed bed resin (Amberlite MB-3, Fisher Scientific Co.). Since the effluent contained radioactive material other than glucose, further purification was carried out by phosphorylation of the glucose, adsorption of the sugar phosphate on an ion-exchange resin, washing the phosphate ester free of contaminants, elution of the ester, and, finally, dephosphorylation. One-half of the deionized solution, containing approximately 200 micromoles of glucose, was brought to pH 7.6 with 0.1 N KOH and treated with 400 micromoles of adenosine triphosphate (ATP), first brought to pH 7.6, 100 micromoles of $MgCl_2$, and 10 mg. of crude yeast hexokinase (8). The mixture was stirred magnetically at 25° C. in a beaker in which external electrodes from a Beckman pH meter were suspended. The reaction was followed by measuring the quantity of 0.1 N NaOH required to maintain the pH, since for every mole of glucose phosphorylated, one mole of acid is produced (8). Following phosphorylation of 82 per cent of the glucose, the mixture was adsorbed on Dowex-1-acetate (the chloride form of the resin is equally satisfactory). The resin was washed with several volumes of water until the effluent was free of radioactivity, and the glucose-6-phosphate was eluted with 0.1 N HCl. The eluate was neutralized with 1.0 N KOH, and the volume reduced to 5.0 ml. by lyophilization. The pH was adjusted to 5.0, and the ester was dephosphorylated by treatment with 10 mg. of acid phosphatase at 37° C. for three hours. The solution was then deproteinized with perchloric acid and deionized as described above. An aliquot of this solution was chromatographed in butanol-acetic acid-water (9), and was found to contain a single radioactive spot on radioautography.

Glucose was degraded by incubation with *Leuconostoc mesenteroides* (10). The fermentation products were converted to $BaCO_3$ by techniques which have been summarized elsewhere (11), and which permit the isolation of each carbon atom of the glucose molecule free of the others. In order to determine whether added glucose-6-phosphate could be degraded by *L. mesenteroides* the sugar phosphate was incubated with a lyophilized preparation of the organism. The lyophilized bacteria fermented glucose actively, but had no discernible effect on the phosphate ester. Thus, the dephosphorylation step was required prior to further degradation of the isolated sugar.

Chemical and enzymatic procedures. Ribose was analyzed by the orcinol procedure (12) and glucose by the method of Nelson (13) and Somogyi (7). Glucose-6-

[1] This investigation has been aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research and from the National Cancer Institute (C-3151), United States Public Health Service.

phosphate was assayed spectrophotometrically with glucose-6-phosphate dehydrogenase and triphosphopyridine nucleotide (TPN) (14).

Materials. Ribose-1-C^{14} was obtained from the National Bureau of Standards through the courtesy of Dr. H. S. Isbell. Crude yeast hexokinase, glucose-6-phosphate dehydrogenase, TPN, and ATP were purchased from Sigma Chemical Co. Glucose-6-phosphate was purchased from Schwarz Biochemical Co.

Radioactivity determinations were carried out with a "micromil" end-window counter with a counting efficiency of approximately 20 per cent. Barium carbonate was counted at "infinite" thickness and all other samples at "infinite" thinness.

RESULTS

Blood glucose samples, obtained 15 minutes and 2 hours following the injection of ribose-C^{14}, had specific activities of 395 and 133 counts per minute (cpm) per mg., respectively.

The degradation data are presented in Table I. Radioactivity was found predominantly in carbons 1 and 3 of the glucose molecule, with the latter more active than the former. The isotope pattern in the sample isolated at 2 hours was virtually identical with that observed in the 15 minute specimen, and very similar to the distribution of radioactivity in mouse (3) and in rat (15) liver glycogen isolated following ribose-1-C^{14} administration (Table I).

The unlikely possibility that trace quantities of radioactive ribose remaining in the circulation could have contributed to the isotope pattern can be ruled out as a result of a previously reported investigation (3). In this study a mixture of unlabeled glucose and ribose-1-C^{14} was degraded by *L. mesenteroides*. No radioactivity was found in carbons 1 or 3, the sites of major isotope concentration in the blood glucose of the patient given ribose-1-C^{14}.

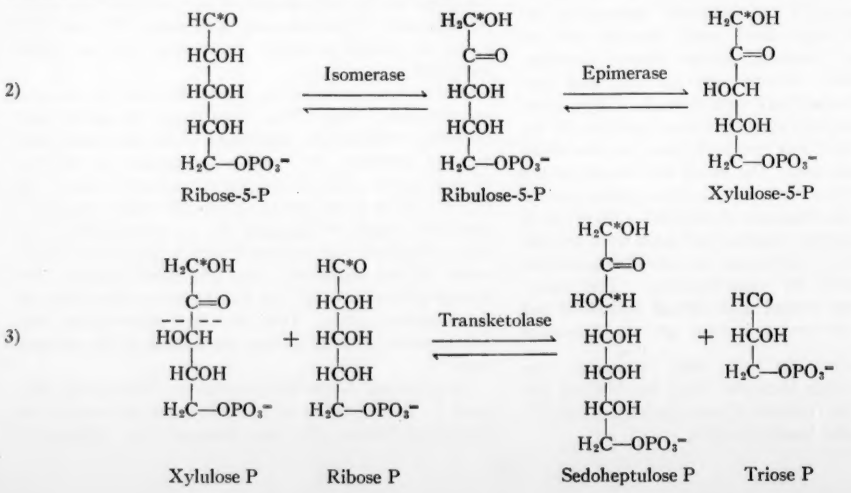
DISCUSSION

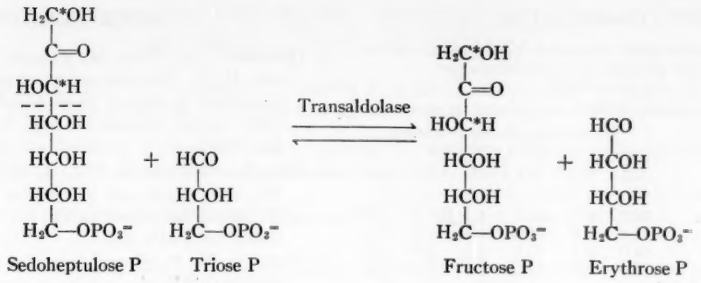
The fraction of ribose carbon which was converted to blood glucose within the 15 minutes following ribose administration may be calculated from the product of the specific activity of the blood glucose [395 cpm per mg.], the blood glucose concentration [90 mg. per 100 ml.], and the glucose space [24 per cent (16) of the patient's body weight, 50 kilograms]. This figure, 4.80×10^6 cpm, which represents 16 per cent of the administered radioactive carbon, is a minimal one, for it does not include the glucose catabolized during the 15 minutes following ribose administration. Six to fifteen per cent of the administered radioactivity was found in liver glycogen of mice given ribose-1-C^{14} (3).

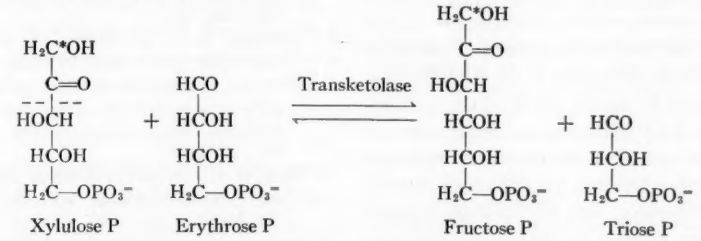
Studies with plants, bacteria and animals suggest a uniformity in the pathway of conversion of ribose to hexose (4). The pentose must first be phosphorylated, and a specific kinase for this reaction has been isolated from mammalian liver (17).

1) Ribose + ATP → ribose-5-phosphate + ADP (adenosine diphosphate)

This pentose phosphate may then be converted to hexose phosphate via the nonoxidative series of reactions of the pentose phosphate pathway:



4) 

5) 

To summarize these reactions, three molecules of ribose phosphate would give rise to two molecules of fructose phosphate and one of triose phosphate. Radioactivity, originally introduced in the first carbon atom of pentose, is denoted by an asterisk in reactions 2 through 5. In reaction 3 the first two carbons of xylulose phosphate are transferred to ribose phosphate. Thus, carbon 1 (C-1) of the product, sedoheptulose phosphate, is derived from the first carbon of one molecule of pentose and carbon 3 from C-1 of the other. Since in reaction 4, the first three carbons of fructose-6-phosphate are derived from the first three carbons of sedoheptulose, then carbons 1 and 3 of hexose originate from carbon 1 of ribose. Hence, if hexose is synthesized from ribose via these reactions, radioactivity introduced in position 1 of ribose should appear in carbons 1 and 3 of hexose. Such was indeed found to be the case in the blood glucose isolated from the patient injected with ribose-1-C^{14} (Table I); this glucose was labeled almost exclusively in carbons 1 and 3. The striking similarity of isotope distribution in this glucose with mouse and rat hexose synthesized from ribose carbon is consistent with ribose to hexose conversion via the same pathway in all three species.

The mechanism which accounts for the predominance of isotope in carbon 3 as compared to carbon 1 has been discussed in detail elsewhere (3). To summarize briefly, it has been shown that fructose-6-phosphate as well as xylulose phosphate may act as the donor of "active glycolaldehyde," the term applied to the two-carbon fragment transferred in the transketolase reaction (reaction 3) (18). If ribose-1-C^{14}-phosphate were the *acceptor* in this reaction and unlabeled fructose phosphate were the *donor*, sedoheptulose-3-C^{14}-phosphate would be the product:

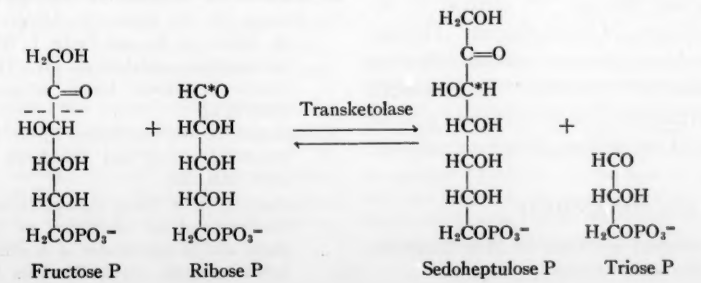
6) 

TABLE I

Distribution of radioactivity in human blood glucose and in animal liver glycogen after administration of ribose-1-C¹⁴

Sample	Per cent total activity in glucose carbon atoms					
	C-1	C-2	C-3	C-4	C-5	C-6
15 minute glucose	30.1	0.9	66.7	1.5	0.4	0.4
2 hour glucose	31.4	1.0	65.4	1.4	0.4	0.4
Mouse glycogen[3]	34.0	3.5	52.0	4.5	3.0	3.0
Rat glycogen[15]	32.7	2.7	58.2	2.5	1.7	2.2

The transfer of the first three carbons of sedoheptulose-3-C¹⁴ to triose phosphate in the transaldolase reaction (reaction 4) would result in the production of hexose-3-C¹⁴-phosphate. Since in our study the ribose was administered in tracer quantity, nonradioactive fructose phosphate, a normal intracellular component, was undoubtedly present in large quantity relative to that xylulose-1-C¹⁴-phosphate derived from ribose-1-C¹⁴ in reaction 2. Hence, the amount of available unlabeled "active glycolaldehyde" (derived from fructose phosphate) was considerably greater than the amount of radioactive "active glycolaldehyde" (derived from xylulose-1-C¹⁴-phosphate). In the transketolase reaction the *acceptor* molecule was ribose-1-C¹⁴, which transferred its radioactivity to position 3 of the product (reactions 3, 4, and 6). The isotope concentration in carbon 1 of the hexose product, however, was diluted to an extent dependent on the proportion of nonradioactive to radioactive "active glycolaldehyde" participating in the reaction. Evidence supporting this hypothesis has been presented in the previously cited report (3).

SUMMARY

Ribose specifically labeled with radioactive carbon was administered to an elderly female with advanced malignant disease. A method is described for the isolation of blood glucose. The isotope pattern in blood glucose isolated following the administration of ribose-1-C¹⁴ is consistent with the transformation of ribose carbon to glucose via the reactions of the pentose phosphate pathway.

ACKNOWLEDGMENT

The valuable technical assistance of Miss Jacqueline Lareau is gratefully acknowledged.

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CHOLESTEROL BIOSYNTHESIS. STUDIES RELATED TO THE METABOLIC ROLE OF SQUALENE [1]

By MAXWELL L. EIDINOFF, JOSEPH E. KNOLL, BENJAMIN J. MARANO, ELLING KVAMME,[2] ROBERT S. ROSENFELD, AND LEON HELLMAN

(From the Divisions of Biophysics and of Steroid Biochemistry, Sloan-Kettering Institute for Cancer Research, Memorial Center, New York, N. Y.)

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Following the demonstration by Langdon and Bloch that squalene is efficiently converted to cholesterol in the tissues of the mouse, there has been considerable experimental activity relating to the role of this compound in cholesterol biosynthesis (1–5). In a study of several aspects of this problem in man, it was found in this laboratory that squalene in human sebum was labeled with radiocarbon following oral administration of acetate containing this isotope (6). Using tritium as an isotopic label, a hydrocarbon fraction containing squalene with high specific activity has been prepared and demonstrated to be a cholesterol precursor in the mouse, and to a significant extent, in the human. In addition, the extent of conversion of squalene along metabolic pathways leading to respiratory carbon dioxide and lipid fatty acids was investigated in the mouse using squalene labeled with radiocarbon.

MATERIALS AND METHODS

Tritium and radiocarbon assay. All tritium assays were made by internal gas phase counting of the hydrogen gas obtained by combustion of the organic compound and complete conversion of the resulting water to hydrogen gas over powdered zinc. The hydrogen gas (admixed with methane to a total pressure of 70 cm. Hg) was counted in the upper portion of the proportional region (7). Radiocarbon assays were made by gas-flow windowless counting in the Geiger-Müller region. In the case of samples of respiratory carbon dioxide with low activity (Tables IV and V), the more sensitive internal gas counting procedures described elsewhere were used (8, 9).

Preparation of hydrocarbon fractions containing squalene-T and assay of total radioactivity. The reaction mixture consisted of 5.0 ml. redistilled squalene (Distillation Products Industries), 5.0 ml. water containing tritium (specific activity, 1 mc. per mM), 0.14 Gm. KOH and 1.0 Gm. powdered platinum catalyst, prepared by the reduction of platinum oxide with ordinary hydrogen gas. After removal of air, the glass ampul containing the reagents was sealed and shaken at 135° C. for 27 hours. In order to remove tritium in relatively labile positions and eliminate contaminating fats, the product was treated with aqueous alcoholic KOH under reflux conditions in a nitrogen atmosphere for four hours, then cooled and extracted with petroleum ether. The latter solution was concentrated and chromatographed on alumina. The hydrocarbon fraction, which was eluted from the column with petroleum ether (60° C.), contained over 99.8 per cent of the tritium activity. The product was stored as a dilute solution in petroleum ether at refrigerator temperatures in order to minimize oxidation. This product (Sample I, the hydrocarbon fraction containing squalene-T) was used in the experiments described in Tables II and III. The specific activity, based on the hexahydrochloride derivative, was 0.5 μc. per mg. squalene. A second tritium-containing hydrocarbon fraction (Sample II) was prepared from Sample I via the thiourea adduct (10). One Gm. of Sample I, admixed with an equal weight of redistilled natural squalene, was shaken with 38 ml. of a saturated solution of thiourea in methanol. The crystalline adduct was allowed to precipitate at refrigerator temperature. After filtering and washing with iso-octane, the precipitate was then decomposed with boiling water. The squalene was extracted with petroleum ether, which was then dried over sodium sulfate and allowed to percolate through an alumina-packed column in order to remove oxygen-containing impurities. The total tritium activity per ml. of Samples I and II was measured by adding 0.10 ml. of a known dilution of these samples to 20 mg. cholesterol in a porcelain combustion boat. The boat was inserted into the combustion tube immediately in order to eliminate loss of squalene by surface film effects during solvent evaporation. After removal of the solvent under reduced pressure, the residue in the boat was combusted in the usual manner. The total voume of hydrogen gas corresponded to at least 99 per cent of the hydrogen content of the sample in the boat. A portion of this gas was then counted.

Determination of percentage of total activity present as squalene (Table I). In the case of Sample I, the assay was based on the specific activity of the squalene hexahydrochloride (M. P., 109° to 20° C.) prepared from a known dilution with redistilled natural squalene by the

[1] This investigation was carried out under contract AT(30-1)-910 with the United States Atomic Energy Commission and supported in part by grants from the National Cancer Institute (C-440) and National Heart Institute, United States Public Health Service.

[2] Present address: Ullevål Hospital, Central Laboratory, Oslo, Norway.

TABLE I

Assay of hydrocarbon fraction containing squalene-T

Sample	Treatment following exchange reaction	Squalene derivative	Percentage of total activity as squalene
I	Saponification and extraction	Mixed hexahydrochlorides	55 ± 4
II	I was purified via thiourea adduct, followed by regeneration and chromatography over alumina	Mixed hexahydrochlorides	68 ± 4
		112° melting point hexahydrochloride	56 ± 3
		Dodecabromide	61 ± 1

method of MacKenna, Wheatley, and Wormall (11). In the case of Sample II, assay was based on the specific activities of the mixed hydrochlorides, the 111 to 112° C. low melting isomer and the dodecabromide prepared as described by MacKenna and co-workers (11). Analysis for dodecabromide: C, 26.17; H, 3.71; Br, 70.2. Calculated: C, 26.3; H, 3.74; Br, 70.0. The results are presented in Table I. On the basis of these derivatives, respectively, the percentage of the total tritium content present as squalene was calculated to be 68, 56 and 61. The remainder of the tritium activity may be present in compounds formed from squalene during the exchange treatment as a result of double bond shifts and other types of isomerization. Evidence for this was obtained from infra-red absorption measurements. The infra-red spectrum of the squalene-T prepared by catalytic exchange was measured using a Perkin-Elmer double beam spectrophotometer. The absorption was very similar to that for redistilled commercial squalene. At 11.29 μ, the tritiated product had a larger percentage absorption relative to the commercial product. An enhanced absorption close to this wavelength had been observed by Dauben and Bradlow (12), and Tomkins, Chaikoff, Dauben, Bradlow, and Srere (13) for squalene regenerated from the hexahydrochloride and for synthetic squalene-C^{14}. These authors concluded that isomeric forms of squalene, different with respect to double bond positions, were responsible for the absorption at this wavelength.

Animal experiments using squalene-T. In Experiments 1 to 4 and 6 (Table III), an aliquot of squalene-T containing about 30 mg. of squalene and an activity of 3×10^7 dpm was mixed with ground Purina Chow and the solvent was removed in a nitrogen stream. The animal was sacrificed 24 hours after the sample was consumed. In Experiment 5, an emulsion of squalene in isotonic saline was prepared by adding water to an equal volume of acetone containing the squalene-T. The acetone was removed by repeated addition of water and evaporation to the original volume. This emulsion was injected into the tail vein and the animal was sacrificed after 24 hours. The mouse liver was homogenized and deproteinized with acetone-alcohol (1:1). An aliquot was removed for cholesterol analysis and the remainder saponified with alcoholic KOH. Following the addition of carrier cholesterol (about 400 mg.) the combined dried ether extracts were concentrated to a small volume. This was chromatographed on alumina and the fractions were eluted with petroleum ether followed by increasing concentrations of benzene. The 100 per cent benzene fraction contained almost all the cholesterol. This was converted to the dibromide, which was debrominated by sodium iodide in ethanol-benzene (14, 15). The cholesterol was recrystallized 2 times from acetone and melted at 148 to 149° C.

Administration of hydrocarbon fraction containing squalene-T in the human (Table II). Petroleum ether was distilled under nitrogen from a solution containing 150 mg. squalene and having an activity of 1.5×10^8 dpm (assayed as squalene) or 2.7×10^8 dpm total tritium activity. The nonvolatile residue was dissolved in 10 ml. sesame oil and homogenized in 100 ml. chocolate milk. This was administered to a 59 year old male, weighing 75 Kg. and of limited life expectancy. The subject had cancer of the prostate and at the time of the experiment was considered to be in good nutritional status. The plasma cholesterol determinations were carried out by a modification of the Sperry-Webb procedure (16).

Preparation of squalene-C^{14}. Acetate-2-C^{14} (500 μc., 0.5 mM) was administered intraperitoneally to a dog that had ingested 20 Gm. natural squalene prior to the experiment. After 40 minutes the animal was sacrificed. The hydrocarbons present in the nonsaponifiable fraction of the liver were obtained by the procedure of Langdon and Bloch (17). Approximately 0.1 per cent of the administered radiocarbon was present in this hydrocarbon eluate. The radiocarbon activity per ml. of the hydrocarbon fraction obtained from the dog liver was measured by gas phase counting of the carbon dioxide from the oxidation of an aliquot (containing a known excess of natural squalene), using the Van Slyke-Folch combustion mixture (18). Another aliquot was mixed with a known excess of squalene, the hexahydrochloride prepared and the specific activity determined by gas phase counting. On this basis, it was found that substantially all the radiocarbon activity was present as squalene (squalene activity/total activity: 1.05 ± 0.04). A lower

TABLE II

Conversion of hydrocarbon fraction containing squalene-T to cholesterol and fatty acid in the human

Time after administration (*hrs.*)	Specific activity of plasma-free cholesterol (*dpm/mg.*)	Specific activity of fatty acids in blood lipids (*dpm/mg.*)
1	18	
3	145	106
7	2,710	156
21	4,760	53
25	4,240	
43	3,720	
79	2,980	

limit to the specific activity of the squalene in the hydrocarbon fraction was established by determining the amount of squalene per unit volume of solution. This quantity was too small to assay accurately by chemical methods. Consequently, the infra-red spectrum was measured in carbon disulfide solution using a Perkin-Elmer double beam spectrophotometer and compared with spectra for known concentrations of redistilled natural squalene. It was found that the specific activity of the squalene-C^{14} was greater than 3 times 10^6 dpm per mM.

Isolation of fatty acids (*Table V*). The nonsaponifiable portion of the alcoholic-aqueous KOH solution was extracted three times with petroleum ether. The latter was washed with small portions of 2N KOH and then with water, and these were combined with the soap fraction. The alkaline fraction was neutralized and the organic solvents (ethanol and traces of petroleum ether) were evaporated off. The aqueous solution was acidified with sulfuric acid and the resulting turbid suspension was placed in a refrigerator to allow the fatty acids to precipitate. The solution was filtered and the filter paper plus residue was continuously extracted with petroleum ether for 16 hours in a Soxhlet apparatus. Controls using stearic acid showed that more than 90 per cent of this fatty acid could be recovered on a semimicro scale. Aliquots of the resulting solution containing the labeled fatty acids were plated out, using aluminum planchets containing a lens paper disc.

RESULTS

Oral administration of hydrocarbon fraction containing squalene-T in the human

The specific activity of plasma-free cholesterol following oral administration of the hydrocarbon fraction containing squalene-T at various intervals following administration of the dose is listed in Table II and plotted in Figure 1. The presence of cholesterol labeled with tritium was readily detectable after one hour. The specific activity reached a maximum in the interval between the 7 and 21 hour points and the curve after 21 hours is roughly comparable to that for plasma-free cholesterol after oral ingestion of the labeled sterol (19). The specific activity of the fatty acids in the blood lipids was measured at 3, 7 and 21 hours (column 3, Table II). At 7 and 21 hours, the fatty acid specific activity was 0.06 and 0.01 of the plasma-free cholesterol, respectively.

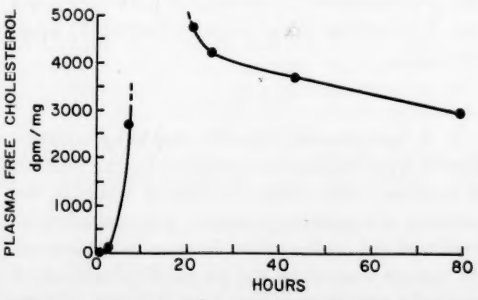

FIG. 1. SPECIFIC ACTIVITY OF PLASMA-FREE CHOLESTEROL FOLLOWING ADMINISTRATION IN THE HUMAN OF HYDROCARBON FRACTION CONTAINING SQUALENE-T

TABLE III

Tritium content of liver cholesterol following administration of hydrocarbon fraction containing squalene-T in the mouse

Expt. no.	Sample administered	Percentage conversion based on: Activity as squalene	Total activity
1*	I	2.1	1.1
2	I	0.75	0.41
3	I	0.63	0.35
4	I	0.90	0.49
5	II	0.25	0.15
6	II	0.10	0.06

* Administered by intravenous injection of emulsion in saline in Experiment 5; by admixture with food in the other experiments.

Administration of labeled squalene in the mouse

a) *Squalene-T*. The liver cholesterol (purified via the dibromide procedure) was assayed. The percentage conversion of squalene to cholesterol in this organ ranged from 0.10 to 2.1 per cent (column 3, Table III).

b) *Squalene-C^{14}*. Following intravenous injection of the squalene-C^{14} in the mouse, cholesterol in the liver and remainder of the carcass as well as the respiratory carbon dioxide was assayed for radiocarbon content (Table IV). At the end of five hours, 3.3 per cent of the administered activity was present as liver cholesterol while less than 1 per cent of the radiocarbon administered was present as cholesterol in the remainder of the carcass. Following oral administration (Table V) approximately 1 per cent of the administered radiocarbon was present as cholesterol, mainly in the liver. Relatively small conversion of the radiocarbon in the administered squalene-C^{14} to respiratory carbon dioxide was found (0.5 and 0.2 per cent, respectively) at the time of sacrifice (Tables IV and V). Approximately 1.5 per cent

TABLE IV

Conversion of squalene-C^{14} to cholesterol and respiratory carbon dioxide in the mouse—Intravenous administration

Compound	Source	Percentage of injected activity
Cholesterol	Liver	3.3
	Remainder of carcass	<1
Carbon dioxide	Collection interval of expired air (*hrs.*)	
	0–0.5	0.02
	0.5–1.0	0.00
	1.0–1.5	*
	1.5–2.0	0.00
	2–3	0.24
	3–4	0.15
	4–5	0.06
	Total	0.47

* Sample lost during analysis.

TABLE V

Conversion of squalene-C^{14} to cholesterol, fatty acids and respiratory carbon dioxide in the mouse—Oral administration

Compound	Source	Percentage of administered activity
Cholesterol	Liver	0.86
	Remainder of carcass	0.07
Lipid fatty acids	Liver	<0.01
	Remainder of carcass	1.5
Carbon dioxide	Collection interval of expired air (*hrs.*)	
	0–0.5	0.01_1
	0.5–1	0.02_2
	1–2	0.03_3
	2–3	0.03_1
	3–4	0.03_1
	4–5	0.03_1
	5–6	0.01_8
	6–7	0.02_1
	Total	0.20

of the administered radiocarbon (Table V) was present in the lipid fatty acids. Each mouse in the experiments summarized in Tables IV and V received 8 times 10^4 dpm radiocarbon contained in less than 11 mg. squalene.

DISCUSSION

The results in the mouse (Table III) served to demonstrate that the squalene-T preparation was a useful precursor material for studies of cholesterol biosynthesis. The oral administration of this precursor in the human (Table II) resulted in a much larger percentage conversion to cholesterol than in the case of the mouse experiments. The extent to which the tritium present in the administered dose was incorporated into cholesterol can be estimated from the circulating cholesterol level together with reasonable assumptions concerning equilibrium of plasma cholesterol with other cholesterol pools. The specific activity at 25 hours was 4.24×10^3 dpm per mg. cholesterol. The plasma cholesterol level was 176 mg. per 100 ml. It can be reasonably assumed (for the purposes of this calculation) that there are: *a*) 43 ml. plasma per Kg. body weight and *b*) rapid equilibration of plasma cholesterol with 3 Gm. red blood cell free cholesterol, 3.8 Gm. free and 0.7 Gm. ester cholesterol in the liver (16). Since the total administered radioactivity was 2.67×10^8 dpm, it follows that 24 per cent of the tritium activity was converted to cholesterol. If it is further assumed that only the squalene-T was utilized, this would correspond to a utilization of over 40 per cent of the administered label. When labeled cholesterol was fed to subjects, it was found that about 12 per cent appeared in the plasma (19). The extent of conversion in the case of squalene as precursor is significantly high. This result is direct experimental evidence for the hypothesis that squalene or a closely related compound is an important intermediate in the synthesis of cholesterol by the human. Using absorption data reported in animal experiments and the results of studies in the human, McGuire and Lipsky had estimated that approximately 20 per cent of liver cholesterol was derived from administered (unlabeled) squalene (20).

SUMMARY

1. A hydrocarbon fraction containing squalene labeled with tritium was prepared by the reaction of squalene with tritium-containing water in the presence of a platinum catalyst. On oral administration of this hydrocarbon fraction in the human, the tritium content of the plasma-free cholesterol reached a maximum within 7 to 21 hours. Calculations based on these data indicated that over 20 per cent of the administered tritium label was con-

verted to cholesterol tritium. Incorporation of the labeled squalene into the liver cholesterol of mice was also demonstrated. These results furnish direct support for the hypothesis that squalene, or a closely related compound, is an important intermediate in the synthesis of cholesterol by the human.

2. The *in vivo* synthesis of squalene from acetate precursor was used to prepare squalene-C^{14} for metabolic studies. On intraperitoneal administration of acetate-2-C^{14} to a dog, approximately 0.1 per cent of the administered radiocarbon activity was present in the liver squalene after one hour. The labeled squalene was administered to mice and the radiocarbon content of cholesterol, fatty acids, and respiratory carbon dioxide measured. Less than 1 per cent of the administered radiocarbon was eliminated as respiratory carbon dioxide in a seven hour period following administration. After this time, the mouse lipid fatty acids contained less than 2 per cent of the radiocarbon in the administered squalene.

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THE GRANULATION OF JUXTAGLOMERULAR CELLS IN RENAL HYPERTENSION, DESOXYCORTICOSTERONE AND POST-DESOXYCORTICOSTERONE HYPERTENSION, ADRENAL REGENERATION HYPERTENSION, AND ADRENAL INSUFFICIENCY [1, 2]

By LOUIS TOBIAN,[3] JANET THOMPSON, ROBERT TWEDT, AND JEANETTE JANECEK

(From the Department of Internal Medicine, University of Minnesota School of Medicine, and the University of Minnesota Hospitals, Minneapolis, Minn.)

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The pathogenesis of renal hypertension remains a mystery. Renal disease can be severe enough to produce terminal uremia without producing hypertension. On the other hand, hypertension can be induced by partial constriction of the renal artery, and the kidney in question may retain good excretory function. The excretory activities of the kidney seem not to be directly related to its hypertensive or antihypertensive functions. It is therefore inviting to consider aspects of renal anatomy other than those directly and obviously concerned with excretion. Such a structure is the juxtaglomerular apparatus. Certain cells of this juxtaglomerular complex containing discrete secretory granules are found in the wall of the afferent glomerular arteriole, just before it joins the glomerulus. Goormaghtigh studied them many years ago in relation to renal hypertension (2). These cells are in a position which would be particularly affected by changes in intraluminal pressure, such as occurs with Goldblatt clamping. In the classic Goldblatt experiment, the renal artery is partially constricted, producing a decrement in blood pressure downstream. Such a decrement in pressure would reduce the stretch of the walls of the entire renal arborization, including the juxtaglomerular cells residing in the arteriolar walls. These cells, then, could be stretch receptors which change their rate of secretion in response to changes in stretch. With this in mind, the juxtaglomerular apparatus was studied in relation to the Goldblatt type of experimental hypertension. If the juxtaglomerular apparatus has any fundamental role in renal hypertension it might also be altered in other forms of experimental hypertension. Therefore, rats with desoxycorticosterone hypertension, postdesoxycorticosterone hypertension, and adrenal regeneration were also studied.

METHODS

This study was greatly facilitated by the use of the staining technique developed by the Hartrofts for the juxtaglomerular (JG) apparatus (3). Each granule is discretely stained in this method. A 40x fluorite oil immersion objective enabled us to see the individual secretory granules much better than the usual 43x achromatic "high dry" objective, yet provided a large field for rapid scanning. Compensating eyepieces (12x) were used in conjunction with this objective. The degree of granulation in the juxtaglomerular apparatus was estimated using the system devised by the Hartrofts (3). Their weighted juxtaglomerular index (JG index) was utilized, giving a total index number per 100 glomeruli. This system is advantageous in that it counts each JG complex and weighs it according to its degree of granulation. Experience indicated that whenever a large number of juxtaglomerular complexes were to be seen, the number of granules per complex were usually also increased. Even though this is a semiquantitative method, it has considerable precision. Two different observers scanning the same slide rarely differed by more than 10 per cent. Figure 1 shows various groups of juxtaglomerular cells.

Renal hypertension was produced in our rats by partially constricting the renal artery, using a silver ribbon clip. This is the method developed by Wilson and Byrom (4) and used extensively in recent years by Floyer (5). In the rat, hypertension can be produced by constricting only one renal artery and leaving the other

[1] This investigation was supported by a grant from the American Heart Association and by Grant No. H2008 from the National Heart Institute, United States Public Health Service.

[2] This article is a full report of studies presented at the meeting of the American Federation for Clinical Research, May, 1957 (1).

[3] This work was started during the tenure of an Established Investigatorship of the American Heart Association.

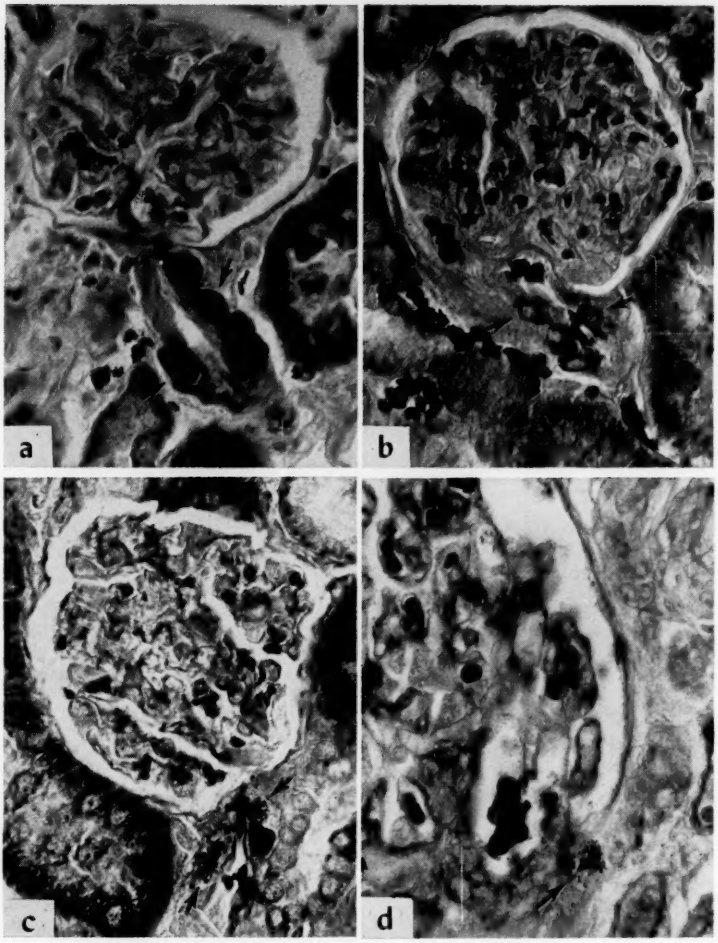

FIG. 1. VARIOUS GROUPS OF JUXTAGLOMERULAR CELLS

In picture a, eight juxtaglomerular (JG) cells loaded with granules are seen in the wall of the afferent arteriole. In b four JG cells laden with granules are seen in the wall of the afferent arteriole. The unstained nuclei of these cells are surrounded by the black granules. In c, two cells are seen which are only moderately filled with granules. Pictures a, b, and c are sections from rat kidney. Picture d shows one granular JG cell in a section from the kidney of a patient with malignant hypertension.

kidney untouched (4, 5). This form of hypertension was utilized in these studies. Floyer's useful terminology is also employed. The "clipped" kidney is one with the renal artery partially constricted; the "untouched" kidney is the unmanipulated kidney opposite to the "clipped" one (5).

The level of arterial blood pressure in rats was determined with the Friedman microphonic method (6). The rats were lightly anesthetized with U.S.P. ether during the blood pressure measurements.

Only male rats were utilized in these experiments. They were all of the Wistar strain except those used in the adrenal insufficiency and adrenal regeneration experiments. Except in the postdesoxycorticosterone acetate (DCA) group, the rats usually weighed between 200 and 400 Gm. Within this range, the JG counts did not noticeably vary with weight or age. The rats at all times were either eating Purina laboratory chow (0.24 per cent sodium) or the special "synthetic" diets mentioned below.

RESULTS

A. *Renal hypertension*

Normal rats. The average juxtaglomerular index for 12 normal Wistar rats weighing between 200 and 300 Gm. is 36, with a standard deviation of ± 8. The mean blood pressure for 90 normal Wistar rats is 99 (S.D., ± 7) mm. Hg (Table I).

Sham operated rats. In nine rats a sham operation on one kidney was performed. The renal artery of this one kidney was dissected out, a silver clip was applied to it, and the clip was then immediately removed and the incision sutured. Five weeks after this sham operation, the operated kidney had a mean juxtaglomerular index of 34 (S.D., ± 12) and the contralateral unmanipulated kidney had a mean JG index of 35 (S.D., ± 13) (Table I).

Rats with unilateral constriction of a renal artery. In sixteen Wistar rats weighing between 150 and 250 Gm., a clip was applied to one renal artery in such a way as to constrict it partially; the other kidney was "untouched." Ten weeks later the blood pressure of these rats was ascertained and eight were found to be definitely hypertensive, with a blood pressure of over 140 mm. Hg (Table II). In these eight rats the "clipped" kidneys had an average JG index of 76, which is significantly higher than that of normal or sham operated kidneys ($p = 0.0001$). The "untouched" contralateral kidneys of these rats showed striking

TABLE I

The mean juxtaglomerular indices for several groups of Wistar rats with renal hypertension along with appropriate control groups*

Group	No. of rats	Mean blood pressure at end of experiment (*mm. Hg*)		Data concerning JG† indices		
				Mean	Range of values	Standard error of the mean
a. Normal Wistar rats	12	99	One kidney examined	36	23 to 53	± 2.5
b. Sham operated rats	9		Sham operated kidney	34	23 to 65	±13.0
			Contralateral untouched kidney	35	21 to 65	±13.7
c. Hypertensive rats 10 weeks after unilateral clipping	8	172	Clipped kidney	76	40 to 129	±10.2
			Contralateral untouched kidney	1	0 to 4	± 0.7
d. Hypertensive rats 6 weeks after unilateral clipping	11	159	Clipped kidney	44	21 to 76	± 5.8
			Contralateral untouched kidney‡			
e. Hypertensive rats 7–14 days after unilateral clipping	7	145	Clipped kidney	46	28 to 63	± 4.1
			Contralateral untouched kidney	9	4 to 14	± 1.4
f. Rats with little or no hypertension 7 weeks after unilateral clipping	13	96	Clipped kidney	48	26 to 80	± 4.3
			Contralateral untouched kidney	6	0 to 20	± 1.9
g. Normal rats on a basic diet containing 0.002% Na	8	110	One kidney examined	48	29 to 62	± 4.0
h. Normal rats on the basic diet plus 0.4% Na	8	111	One kidney examined	30	19 to 54	± 3.9
i. Rats with unilateral clipped kidney on a basic diet containing 0.002% Na	22	169	Clipped kidney	66	39 to 98	± 3.5
			Contralateral untouched kidney	6	0 to 33	± 1.8
j. Rats with unilateral clipped kidney on the basic diet plus 0.4% Na	13	181	Clipped kidney	58	22 to 112	± 7.8
			Contralateral untouched kidney	1	0 to 4	± 0.3

* Both kidneys were present in all rats.
† Juxtaglomerular.
‡ This kidney was left intact for subsequent studies (Group b, Table III).

degranulation of the juxtaglomerular apparatus. From a normal value of 36 the index fell to an average of 1 in these kidneys, and the JG index ranged from 0 to 4, far below normal JG indices. These findings are consistent with observations by others that renal ischemia may be associated with an increase in the number of granular JG cells (2, 7) and also with observations that the contralateral "untouched" kidney has a great decrease in the granular JG cells (8, 9).

In another group of hypertensive rats with a similar operation, the ischemic kidney was examined six weeks after constricting the renal artery. The JG index averaged 44 in these kidneys, a value not as high as was seen in constricted kidneys 10 weeks after clipping (Table I).

Another group of seven hypertensive rats was sacrificed 7 to 14 days after a similar constriction of only one renal artery. The "clipped" kidneys had an average JG index of 46 with a range of 28 to 63, and the contralateral "untouched" kidney had a mean JG index of 9 with a range of 4 to 14. Even this brief interval after clipping one renal artery allowed for considerable degranulation of the "untouched" kidney and hypergranulation of the ischemic kidney (Table I).

Thirteen other rats had the same clipping procedure as the foregoing groups of rats. However, they failed to develop hypertension during seven weeks of constriction of one renal artery and all had blood pressures under 110 mm. Hg. Even these rats had significant hypergranulation of the ischemic kidney. In this group the mean JG index of the constricted kidney was 48 (S.D., ± 15), with values ranging from 26 to 80. The "untouched" kidney had a mean JG index of 6 (S.D., ± 6), with values ranging from 0 to 20 (Table I). Even though the "untouched" kidney here showed considerable degranulation, it was not nearly as complete as that seen in truly hypertensive rats with a similar operation.

The degranulation of the "untouched" kidneys after unilateral clipping cannot be the result of renal hypertrophy, since rats with a single fully hypertrophied kidney have normal JG indices (Table III).

TABLE II

The juxtaglomerular indices for eight individual hypertensive rats 10 weeks after a clip had been applied to one renal artery

Rat no.	Blood pressure at end of experiment (*mm. Hg*)	JG* index of ischemic kidney	JG index of contralateral untouched kidney
1	163	68	0
2	174	105	0
3	175	129	3
4	185	40	0
5	150	53	1
6	185	68	4
7	178	62	0
8	168	84	1
Mean values for the group	172	76±27†	1±2

* Juxtaglomerular.
† ± Indicates standard deviation.

TABLE III

The mean juxtaglomerular indices for certain groups of Wistar rats with only one kidney present, including groups with past or present hypertension and control groups

Group	No. of rats	Mean blood pressure at end of experiment (*mm. Hg*)	Data concerning JG* indices		
			Mean	Range of values	Standard error of the mean
a. Normal rats with one kidney, on Purina chow	11		34	9 to 50	±3.6
b. Previously hypertensive rats that became normotensive 6 weeks after the excision of a clipped kidney (eating Purina chow)	11	96	28	16 to 39	±2.2
c. Rats with one clipped kidney on a basic diet containing 0.002% Na	14	174	55	37 to 82	±4.3
d. Rats with one clipped kidney on the basic diet plus 0.4% Na	8	184	45	27 to 94	±7.5

* Juxtaglomerular.

Partial constriction of the renal artery of degranulated kidneys. In five rats, one renal artery was partially constricted with the subsequent development of hypertension. As noted above, the "untouched" contralateral kidney seven weeks later has almost complete degranulation of the JG cells with an average JG index of 1, and values ranging from 0 to 4. At this time the renal artery of this degranulated "untouched" kidney was also "clipped." Four weeks later both kidneys were examined. In two of the five rats, the kidney which may be assumed to have been previously degranulated was still degranulated despite the clipping procedure. However, in the three other rats the previously degranulated kidney had definitely regranulated as a result of the second clipping operation. The JG indices were 36, 28, and 14 in the previously degranulated kidneys of these rats.

The effect of a low sodium diet on the granulation of the juxtaglomerular apparatus. Three experiments were done to assess the influence of the amount of sodium in the diet on the degree of juxtaglomerular granulation. In these studies the diet consisted of purified materials, including 20 per cent casein, 70 per cent sucrose, 5 per cent corn oil, 5 per cent sodium-free salt mixture made with reagent-grade chemicals, and vitamin supplementation. This constituted our "low sodium diet" and contained 0.002 per cent sodium. Our control diet with 0.4 per cent sodium was made by adding pure sodium chloride to the basic low sodium diet. Water was given *ad libitum.*

The two diets were offered to two groups of normal Wistar rats. A group of eight rats on the diet containing 0.4 per cent sodium for five weeks had a mean JG index of 30 (S.D., ± 10). The group of eight rats on the diet containing 0.002 per cent sodium for five weeks had a mean JG index of 48 (S.D., ± 10). Thus the rats on a very low sodium intake had a significantly higher degree of JG granulation ($p = 0.001$). This confirms the earlier observations of the Hartrofts (3) (Table I).

The next experiment deals with hypertensive rats with a clip on one renal artery, the other kidney remaining "untouched." Sixteen weeks after the clip was applied, these hypertensive rats were divided into two groups which were evenly matched for blood pressure. These two matched groups were placed on the experimental diets described above for an additional eight weeks. There was a slight reduction in blood pressure in the group of 22 rats on the 0.002 per cent sodium diet. Before going on the diet, the mean blood pressure of this group was 177 mm. Hg. After eight weeks on the diet, this mean had fallen to 169 mm. Hg. The other group of 13 hypertensive rats on the 0.4 per cent sodium diet had an initial blood pressure of 173 mm. Hg which rose to 180 mm. Hg while on this diet. The group of rats on the 0.4 per cent sodium diet had the usual almost complete JG degranulation in the "untouched" kidney. The mean JG index of the "untouched" kidney in this group was 1 and the highest index for an individual rat was 4. On the other hand, the average JG index of the "untouched" kidney was 6 in the rats on the 0.002 per cent sodium diet, and 7 rats out of the 22 in this group had indices higher than 4. Three rats of this group had indices higher than 17 in the "untouched" kidney. This would be a very unusual finding in operated hypertensive rats on higher sodium intakes. The difference in granulation of the "untouched" kidney between these two groups is highly significant ($p = 0.005$). The diet containing very little sodium unquestionably increased the JG granulation in these "untouched" kidneys. Moreover, the slight reduction in blood pressure resulting from the low sodium intake probably does not account for the increased granulation, since all hypertensive rats on the low sodium diet had a blood pressure higher than 140 at the end of the experiment (Table I).

Varying the amount of sodium in the diet did not greatly influence the JG granulation of the "clipped" kidneys in these rats. The group on 0.4 per cent sodium in the diet had a mean JG index of 58 in the "clipped" kidney, and the group on the 0.002 per cent sodium diet had a mean JG index of 66. The "clipped" kidneys of both groups are hypergranulated compared to a normal kidney but are not significantly different from one another (Table I).

Other rats were made hypertensive by applying a clip on one renal artery and excising the contralateral kidney. All the rats studied had blood pressures over 140 mm. Hg. Sixteen weeks after the operations, they were divided into two groups evenly matched for blood pressure. One group was placed on the 0.002 per cent sodium diet and

TABLE IV

A comparison of the juxtaglomerular indices in adrenalectomized and in normal Sprague-Dawley rats

Group	No. of rats	Mean JG* index	Range of values	Standard error of the mean†
a. Normal rats on Purina chow and tap water	12	30	18 to 40	±1.9
b. Adrenalectomized rats on Purina chow and 1% saline	12	52	7 to 83	±7.2

* Juxtaglomerular.
† p value of the difference between these means: 0.004.

the other on the 0.4 per cent sodium diet for five weeks and they were then killed. The blood pressure of all the rats on either diet remained above 140 mm. Hg at the end of the experiment. The 8 rats on the 0.4 per cent sodium diet had a mean JG index of 45, while the 14 rats on the 0.002 per cent sodium diet had a mean JG index of 55. This difference was not statistically significant ($p = 0.3$) (Table III).

The effect on the juxtaglomerular apparatus of "curing" unilateral renal hypertension with a unilateral nephrectomy. Eleven rats were made hypertensive with blood pressures over 140 mm. Hg by clipping one renal artery as described above. The average blood pressure of this group was 159 mm. Hg seven weeks after the operation. From previous experience, we know that the JG apparatus of the "untouched" kidney is virtually degranulated seven weeks after the original clipping operation in hypertensive rats. At this time the "clipped" kidney was excised. Every rat had a decided fall in blood pressure following this procedure. Ten out of the 11 rats had blood pressure levels under 115 mm. Hg two weeks after the removal of the "clipped" kidney. By six weeks after removal of the "clipped" kidney, all the blood pressures were under 111 mm. Hg with an average of 96 mm. Hg. Thus all rats eventually had a blood pressure well within the normal range. When the lone kidney of these normotensive rats was examined, it had an almost complete regranulation of the JG apparatus with an average JG index of 28 and values ranging from 16 to 39 (Table III). The lone kidney of three of these rats that had become normotensive was seen two weeks after the "clipped" kidney had been removed. The JG indices in these three were 32, 23, and 24, showing that regranulation was almost complete in this short interval.

While it is obvious that regranulation occurs in the JG apparatus of these rats when they change from a hypertensive to a normotensive state, it is of interest to compare the granulation in these lone kidneys with the JG granulation of rats that have had one kidney excised without having undergone any clipping operations.

Eleven Wistar rats had one kidney excised and two months were allowed to elapse for a new equilibrium to be established. The lone hypertrophied kidney of these rats was then studied. The average JG index in this lone kidney was 34 (S.D., ± 11). Thus the regranulated JG complexes of the previously hypertensive rats are not greatly different from those of simple one kidney rats. Moreover, these simple one kidney rats have a JG granulation quite similar to that seen in completely normal rats (Table III).

B. *Adrenal insufficiency*

A number of male Sprague-Dawley rats were randomly divided into two groups with 12 rats in each group. The control rats in Group A with intact adrenals ate Purina laboratory chow and drank tap water *ad libitum*. The rats in Group B underwent a complete bilateral adrenalectomy and subsequently were allowed to drink only a 1 per cent saline solution. They were also eating Purina laboratory chow. All of these rats were killed 18 days after the adrenalectomy of the rats in Group B. The mean JG index in the normal rats of Group A was 30 (S.D., ± 6). The mean JG index in the adrenalectomized rats of Group B was 52 (S.D., ± 24). This difference was highly significant with a p value of 0.004. These results are

similar to those found by Dunihue in adrenalectomized cats (10) (Table IV).

C. *Rats treated with desoxycorticosterone in conjunction with either high or low sodium diets*

Twenty-nine rats were given a subcutaneous injection of 125 mg. of desoxycorticosterone trimethylacetate (DCTMA).[4] This injection was repeated in four weeks. One group with 10 of these rats was fed a "synthetic" diet containing only 0.002 per cent sodium (described above), and water *ad libitum*. Another group with 19 of these rats was fed this same diet with 0.4 per cent sodium added (described above) and was allowed to drink nothing but 1 per cent sodium chloride. A control group that was fed the sodium-deficient diet but did not receive DCTMA can also be compared with the above groups.

All rats were killed two and a half weeks after the second injection of DCTMA. The group on DCTMA and a high sodium intake had a virtual disappearance of the JG granulation. Their average JG index was 0.3 with a range of 0 to 3. Sixteen out of the 19 rats had JG indices of 0. All of these rats had some elevation of blood pressure at the end of the experiment with an average final blood pressure of 142 mm. Hg. On the other hand, the group of rats that received DCTMA and a diet very low in sodium did not show this extreme JG degranulation. The mean JG index was 17 for the group. None of these rats on the low sodium diet became hypertensive during the course of the experiment, and the average blood pressure for the group was 99 mm. Hg at the end of the experiment. These results confirm the previous observations of the Hartrofts (11) (Table V).

The rats receiving DCTMA in conjunction with a diet low in sodium had a much lower JG index than the control group receiving the diet only. This was true in spite of the fact that their blood pressure was lower than that of the sodium-deficient control group. This would seem to indicate that DCTMA alone has a significant ability to cause degranulation of the JG cells independent of hypertension (Table V).

D. *Postdesoxycorticosterone hypertension*

It is well known that desoxycorticosterone in conjunction with a generous intake of sodium will produce hypertension in rats. An example of this effect is demonstrated above. It has also been shown that in certain rats this hypertension may persist long after completion of desoxycorticosterone absorption and while the rats are taking in the usual amounts of sodium (12). This type of hypertension has been called "postdesoxycorticosterone" hypertension and is a prime example of the principle that a hypertension may persist long after its original cause has been removed.

To produce this type of hypertension a 25 mg. desoxycorticosterone acetate pellet was implanted subcutaneously in each of 150 male weanling rats. Another similar pellet was implanted five weeks

[4] We wish to thank Dr. Robert Gaunt of the Ciba Pharmaceutical Company for generously supplying the desoxycorticosterone.

TABLE V

A comparison of the juxtaglomerular indices in Wistar rats receiving desoxycorticosterone and either a high or low sodium intake

Group	No. of rats	Mean blood pressure at end of experiment (*mm. Hg*)	Data concerning JG* indices		
			Mean	Range of values	Standard error of the mean
a. Rats receiving DCTMA† and 1% NaCl in food and water	19	142	0.3	0 to 3	±0.1‡
b. Rats receiving DCTMA and a very low sodium diet	10	99	17	0 to 51	±6.2§
c. Control rats receiving a very low sodium diet	8	110	48	29 to 62	±4.0

* Juxtaglomerular.
† Desoxycorticosterone trimethylacetate.
‡ p value of the difference between groups a and b: 0.009.
§ p value of the difference between groups b and c: 0.00006.

TABLE VI

A comparison of the juxtaglomerular indices in "postdesoxycorticosterone" rats with either elevated or normal blood pressure

Group	No. of rats	Mean blood pressure at end of experiment (*mm. Hg*)	Data concerning JG* indices		
			Mean	Range of values	Standard error of the mean†
a. Post-DCA‡ rats with hypertension	17	160	7	1 to 15	±1.0
b. Post-DCA rats with normal blood pressures	16	104	25	8 to 50	±3.1

* Juxtaglomerular.
† p value of the difference between these means: 0.0000001.
‡ Desoxycorticosterone acetate.

later. The rats were all drinking a 1 per cent sodium chloride solution for six months after the first pellet implantation. Most of them became hypertensive during this period. They were then offered tap water for the next four and a half months, at which time the experiment was terminated. The blood pressure of each rat was determined twice during the last week of the experiment, and the average of these readings was considered the blood pressure for each rat. When the rat was killed, the entire subcutaneous area of the back was examined to be certain that both pellets had been completely absorbed. In two rats the remnant of a pellet was seen and these rats were excluded from the study. This was done to insure that all the rats were indeed in the "postdesoxycorticosterone" phase. Seventeen rats remained moderately to severely hypertensive after the pellets had completely disappeared. The blood pressure was above 140 in every rat of this group with a mean of 160 mm. Hg. Many more rats had very mild hypertension with blood pressures between 120 and 140 mm. Hg. Sixteen rats had blood pressures in the normal range (below 118 mm. Hg) in the postdesoxycorticosterone period. The mean blood pressure of this group was 104 mm. Hg. The JG granulation of the hypertensive group and the normotensive group was compared. In the normotensive group the mean JG index was 25 (S.D., ±12). In the hypertensive group the mean JG index was 7 (S.D., ±4). This difference was quite significant ($p = 10^{-7}$) (Table VI).

E. *Experimental hypertension in rats associated with adrenal cortical regeneration*

Skelton has recently demonstrated a form of experimental hypertension in rats which appears during the regeneration of adrenal cortical tissue following adrenal enucleation (13). In creating this experimental situation, we removed one kidney and one adrenal in male weanling Holtzmann rats. The contralateral adrenal capsule was nicked, and the medullary and cortical tissue was gently pressed out through the nick leaving only the capsule and a thin layer of adrenal cortical cells subjacent to it. These rats were then given a 1 per cent saline solution to drink. Many of them subsequently developed hypertension. A control group of weanling rats with intact adrenals was also included in the experiment. The rats in this control group had one kidney excised and were drinking only a 1 per cent saline solution. Half of the rats with the single regenerating adrenal had a blood pressure over 140 mm. Hg six weeks after their initial operation. The average blood pressure of these nine rats at this time was 171 mm. Hg. Their average JG index was 7. The control group had a mean blood pressure of 121 mm. Hg (S.D., ±7), and an average JG index of 20. The rats with the hypertension of adrenal regeneration had a significantly lower JG index than the operated control rats ($p = 0.0004$) (Table VII). In many ways the hypertension of adrenal regeneration resembles the hypertension associated with desoxycorticosterone. However, the JG granules in desoxycorticosterone hypertension practically disappear, whereas they are definitely present, even if in diminished numbers, in the hypertension of adrenal regeneration.

DISCUSSION

Unfortunately these studies do not delineate the exact function of the JG apparatus. The granular cells in it have all the appearances of secretory cells, even when seen with the electron microscope

TABLE VII

The juxtaglomerular indices in rats with hypertension of adrenal regeneration and in control rats with intact adrenals*

Group	No. of rats	Mean blood pressure at end of experiment (*mm. Hg*)	Data concerning JG† indices		
			Mean	Range of values	Standard error of the mean‡
a. Rats with adrenal regeneration hypertension	9	171	7	0 to 16	±1.7
b. Control rats with intact adrenals	9	121	20	10 to 33	±3.0

* All rats in this study had one kidney excised and were drinking a 1 per cent saline solution throughout the experiment.

† Juxtaglomerular.

‡ p value of the difference between these means: 0.0004.

(14). It is reasonable to assume that the secretory state associated with hypergranulation is the opposite of that associated with degranulation. It is not even certain whether the degranulation of the JG apparatus is associated with rapid secretion or with a cessation of secretion. However, the state of granulation varies greatly in certain hypertensive states as well as with variations in sodium intake and adrenal function. It has been thought by some that the JG granules may contain renin. No direct proof of this exists, but Peart, Gordon, Cook, and Pickering have shown in the rabbit that renin exists only in or around glomeruli and not in purely tubular areas (15). Hence it is possible that the hypothesis is correct.

These juxtaglomerular secretory cells are abundant in all mammalian species, including man (Figure 1). They have also been seen in lower vertebrates such as the frog (16). These cells in the wall of the afferent arteriole are next to the macula densa, a specialized area of cells in the wall of the distal convoluted tubule which is always near the vascular pole of the glomerulus (17). There is also a rich network of nerves surrounding all the specialized juxtaglomerular structures (18). It is well known that the mammalian kidney has an uncannily accurate ability to keep renal blood flow and glomerular filtration constant in the face of widely varying perfusion pressures (19). Renal vascular resistance can make tremendous adjustments to maintain this autoregulation of blood flow and filtration, even when all vasomotor nerves leading to the kidney have been severed and under conditions of local adrenergic blockade with Regitine® (20). These changes in renal resistance are much more responsive to changes in perfusion pressure rather than to changes in blood flow (20). The whole fabric of evidence indicates that the main changes in resistance which accomplish renal autoregulation can be reasonably assigned only to the preglomerular arteriole. Recent experiments indicate that the cell separation theory cannot account for more than a small part of the changes in resistance occurring during autoregulation (20). As mentioned above, the granular JG cells are in a perfect anatomic situation to sense changes in perfusion pressure. Being in the wall of the arteriole, they could easily sense changes in the stretch of the wall and could then secrete more or less of their hormone in response to this stimulus. Changes in perfusion pressure would also tend to alter the glomerular filtration rate. Such a change in filtration rate might somehow be sensed by the specialized cells of the macula densa in the distal tubule. Since the cells of the macula densa are quite close to the JG secretory cells, without even reticular fibers separating them (21), the JG cells might change their rate of secretion in response to a change in glomerular filtration. In either case, this substance might act primarily on the sphincter of small smooth muscle cells located in the afferent arteriole in the region between the JG secretory cells and the glomerular capillary tuft. Both Clara (22) and Appelt (23) have described this sphincter situated at the entrance into the glomerulus. The state of contraction of the cells in this micromuscular sphincter could provide the fine

adjustment of resistance to blood flow that would have to be present on the upstream side of the glomerular tuft in order to have autoregulation of both renal blood flow and filtration.

The data obtained in our study partly support some of this speculation. In situations where the renal arterial bed is fully exposed to hypertensive blood pressures, the granulation of the JG cells tends to be decreased. Examples of this can be found in the "untouched" kidney of unilateral renal hypertension, in post-DCA hypertension, in acute DCTMA hypertension, and in the hypertension of adrenal regeneration. When the blood pressure in the renal arterial bed is lowered, the JG cells tend to increase their granulation. Examples of this can be found in the kidneys of completely adrenalectomized rats, in the various "clipped" kidneys of rats where a clip on the renal artery of either a normal or an "untouched" kidney decreases the pressure distal to the clip, and especially in the "untouched" kidney of rats in whom renal hypertension has been "cured" by excising a "clipped" kidney. Wide variations of sodium intake could also conceivably operate in this manner. High intakes of sodium tend to increase blood pressure and blood volume; exceedingly low intakes tend toward a low blood pressure and blood volume. Either extreme would be likely to produce some change in the stretch of the JG cells.

Whether or not the substance in the granules of the JG cells influences local renal circulation, the question also arises as to whether it influences the caliber of arterioles throughout the systemic circulation. If it did, it could be very important in producing the increased generalized peripheral resistance of arterial hypertension. In hypertension it could act either by producing an overabundance of a pressor substance or by failing to secrete the normal amount of a depressor substance. Since hypertension can be produced by removing both kidneys (24, 25), the latter concept is possibly more attractive. If the substance of the JG cells had a role in renal autoregulation as well as an effect on systemic arterioles, its effect in the hypertension of renal "ischemia" would have to be the opposite for the two different sets of arteriolar smooth muscle. In partial constriction of a renal artery, autoregulation would demand a relaxation of the preglomerular sphincter; while at the same time a generalized constriction of arteriolar lumina would be occurring throughout the systemic circulation.

It is difficult to explain the partial degranulation of the JG cells in the "untouched" kidney of rats whose opposite kidney has been clipped without the development of hypertension. Ninety-five per cent of rats with unilateral clipping develop at least a slight elevation of blood pressure, and these particular rats represent the exceptional 5 per cent. Since one kidney has been clipped, these rats probably have a tendency to become hypertensive. It may be that the JG cells of the "untouched" kidney in this group of rats became degranulated in response to this tendency to hypertension; and their process of degranulation, if it represented the release of a depressor substance or the failure to release the usual amount of a pressor substance (see above), might actually be opposing this hypertensive tendency and thereby helping to maintain the normotensive state. This would imply that the JG cells of the "untouched" kidney were exerting some sort of antihypertensive activity. If this antihypertensive mechanism became exhausted, similar to exhaustion of the pancreatic β-cells, the tendency to hypertension would be converted to actual hypertension. We need much more evidence before this idea can be anything more than pure speculation.

With our present lack of information, we could be completely overlooking even more important determinants of juxtaglomerular granulation. In any case, the JG cells do undergo marked changes in granulation in various forms of experimental hypertension. Their exact role in these experimental models deserves further study.

SUMMARY

The juxtaglomerular index, which indicates the degree of granulation of cells in the juxtaglomerular aparatus, averages 35 in normal Wistar rats. When hypertension is produced by partially constricting one renal artery, the juxtaglomerular granules double in the ischemic kidney and virtually disappear in the "untouched" contralateral kidney. If the ischemic kidney is then excised seven weeks later, the hypertension disappears

and juxtaglomerular granules reappear in normal abundance in the previously completely degranulated contralateral kidney. After partial constriction of one renal artery, the juxtaglomerular granules in the opposite kidney disappear; but if a constriction is then placed on the artery of this degranulated contralateral kidney, the juxtaglomerular granules reappear. Another group of rats with one ischemic kidney and a contralateral degranulated kidney was placed on a sodium deficient diet. In half the rats, this diet caused the juxtaglomerular granules to reappear in the previously degranulated kidney. This diet also increased granulation in normal rats. Partially constricting the renal artery in a rat with only one kidney will produce hypertension, and the granulation of the lone ischemic kidney remains slightly above normal.

Adrenalectomy doubles the juxtaglomerular index; desoxycorticosterone and salt cause the granules to disappear while concomitantly producing hypertension.

Some rats that had received desoxycorticosterone pellets remained hypertensive long after all pellets had been absorbed. The mean juxtaglomerular index for this group was 7, compared to a value of 25 in a similarly treated group that had become normotensive following the absorption of the desoxycorticosterone pellets.

Hypertension was produced in other rats by removing one adrenal and enucleating the other. Both the hypertensive and the control groups had one kidney removed and were drinking saline solution. The hypertensive group with adrenal regeneration folowing enucleation had a juxtaglomerular index of 7, while the control group with intact adrenals had an index of 20.

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OBSERVATIONS ON THE HETEROGENEITY OF MALIC AND LACTIC DEHYDROGENASE IN HUMAN SERUM AND RED BLOOD CELLS [1]

By ELLIOT S. VESELL AND A. G. BEARN

(From the Rockefeller Institute for Medical Research, New York, N. Y.)

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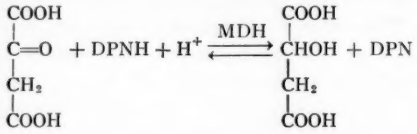
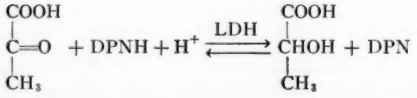
The enzyme malic dehydrogenase (MDH)[2] catalyzes the conversion of oxalacetic acid to malic acid. It resembles lactic dehydrogenase (LDH) in acting on an α-keto acid substrate and in being a pyridine nucleotide dependent dehydrogenase:





It is of interest that Vallee has offered evidence that both rabbit muscle LDH and pig heart MDH are zinc enzymes (1, 2). Reports of elevated serum levels of LDH in cases of myocardial infarction and leukemia led to an earlier study (3) which demonstrated that normal human serum separated electrophoretically contained three enzymes with LDH activity and not one as had been anticipated. In an attempt to elucidate the nature of these three enzymes, an examination of the chemically analogous MDH system was undertaken. The results indicate that MDH activity is also found in three discrete electrophoretic fractions of the serum proteins, two of which closely resemble the corresponding LDH activity peaks.

METHODS AND MATERIALS

This study employed sera obtained from 14 subjects composed of 10 controls (2 healthy young men and 8 patients with such diseases as disseminated lupus erythematosus, cirrhosis, Wilson's disease, and hepatitis), 2 cases of myocardial infarction and 2 cases of chronic lymphatic leukemia.

The method of zone electrophoresis, using a starch supporting medium in barbital buffer, pH 8.6, with an ionic strength of 0.1 or 0.05, was used to separate the serum proteins (4). After electrophoresis the starch block was cut into half inch segments, the protein eluted with 5.0 ml. buffer and its concentration determined by a modification of the Folin-Ciocalteau procedure (4). An aliquot of 2.5 ml. from each eluate was then assayed for MDH activity spectrophotometrically by the method of Wacker, Ulmer, and Vallee (5). Each eluate was incubated for 20 minutes at room temperature with 0.2 ml. of 0.003 molar DPNH. The mixture was transferred to a Beckman cuvette of 1 cm. path length; 0.1 ml. of 0.0076 molar oxalacetate was added, and the decrease in absorption at 340 mμ was measured in the Beckman spectrophotometer. Readings were obtained every 30 seconds for 3 minutes. One unit of dehydrogenase activity was defined as a decrease in the optical density at 340 mμ of 0.001 per minute. Under these conditions, the contribution to the observed MDH activity by the simultaneous measurement of LDH, caused by spontaneous decarboxylation of oxalacetate to form pyruvate, was found to be negligible. Lactic dehydrogenase obtained from rabbit muscle (Worthington laboratories) was found to have insignificant quantities of activity when oxalacetate was used as substrate instead of pyruvate.

In several experiments, a 2.5 ml. sample of an hemolysate prepared from normal human red cells (6) was fractionated electrophoretically and an aliquot of the eluate was assayed for MDH activity. To determine the pH optima of the three peaks, 0.3 ml. was removed from the tube containing the maximum MDH activity, and added to tubes containing barbital buffers varying in pH from 7.4 to 9.2, all at 0.1 ionic strength. MDH activity was then reassayed.

The effect of the inhibitors, ethylenediaminetetraacetic acid and *p*-chloromercuribenzoate, on each of the activity peaks was studied according to the method outlined below. Immediately after the serum fractions had been assayed for enzymatic activity the inhibitor was added, the solution thoroughly mixed and the activity redetermined. The decline in activity between the two measurements was attributed to the action of the inhibitor. The final concentrations of ethylenediaminetetraacetic

[1] These studies were supported, in part, by a grant-in-aid (A-1542) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health.

[2] The abbreviations used in this paper are as follows: MDH = malic dehydrogenase; LDH = lactic dehydrogenase; DPN = diphosphopyridine nucleotide; DPNH = diphosphopyridine nucleotide reduced.

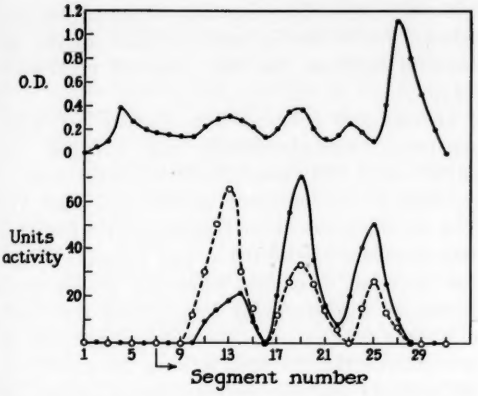


FIG. 1. DISTRIBUTION OF MALIC AND LACTIC DEHYDROGENASE ACTIVITY IN NORMAL HUMAN SERUM

Upper curve illustrates an electrophoric pattern of normal human serum. Lower curves illustrate distribution of malic and lactic dehydrogenase activity in serum fractions. The lactic dehydrogenase activity is indicated by the solid line and malic dehydrogenase activity by the broken line.

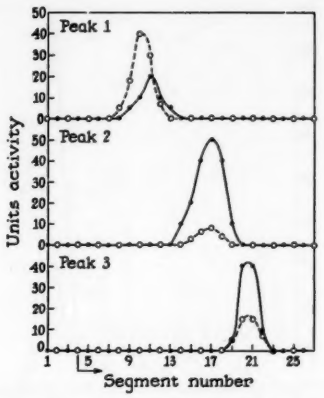


FIG. 2. THE PATTERN OBTAINED WHEN EACH HEMOLYSATE PEAK, AFTER HAVING BEEN ISOLATED FROM A STARCH BLOCK, WAS RERUN ON A SECOND BLOCK

The lactic dehydrogenase activity is indicated by the solid line and malic dehydrogenase activity by the broken line.

acid and *p*-chloromercuribenzoate were 6×10^{-5} M and 1×10^{-4} M, respectively.

The experiments outlined above for serum MDH were also performed on serum LDH by substituting the substrate sodium pyruvate for sodium oxalacetate.

RESULTS

Each of the 14 sera separated electrophoretically and assayed for MDH and LDH activity revealed three main loci of activity (Figure 1). One was found in the β-globulin (peak 1); a second peak of activity was found in the α_2-globulin (peak 2); and the third occurred between the α_1-globulin and the albumin, hereafter referred to as the α_1 peak (peak 3). Following electrophoresis, between 80 and 95 per cent of the MDH activity of the original serum was recovered from the starch block. An average of 9 per cent of the total activity remained adsorbed of the origin. However, the percentage varied widely and when fresh nonhemolyzed serum was used, the amount adsorbed at the origin was extremely small.

In the 10 control subjects (Table I), each peak of MDH activity contained a relatively constant percentage of the total activity found in serum. Forty-six per cent of the total MDH activity was found in the β-globulin, 26 per cent in the α_2-globulin, 19 per cent in the α_1 peak and 9 per cent at the origin. The distribution of LDH activity among the three peaks differed from that of MDH. The α_2 peak contained the largest percentage of LDH activity; the β peak had the largest percentage of MDH activity. Each of the three major components of MDH and LDH activity isolated was rerun under similar conditions and was found to have the same mobilities as in the starting material (Figure 2).

A comparison of the mobilities of the activity peaks of LDH and MDH revealed that the α_1 peaks correspond closely as do the α_2 peaks. However, the peak of LDH activity in the β-globulin was found to migrate slightly faster than the peak of MDH activity (Figures 1, 2, 3, and 4).

Experiments in which phosphate buffer of pH 7.5 and 0.1 ionic strength was used, instead of barbital buffer of pH 8.6, did not demonstrate any alteration in the correspondence of the α_1 and α_2 MDH and LDH activity peaks.

With sodium malate as substrate and DPN as coenzyme, it was found that the location of the three enzymes in the serum fractions was unchanged.

A parallel increase of the α_1 peak of both MDH and LDH was observed in sera from two patients with acute myocardial infarction (Figure 3),

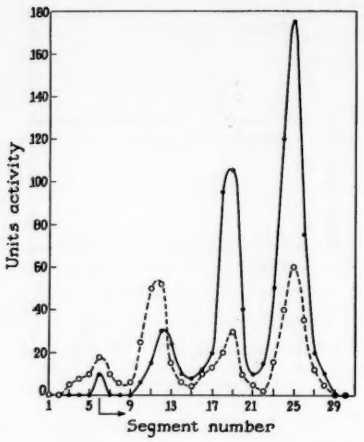


FIG. 3. DISTRIBUTION OF MALIC AND LACTIC DEHYDROGENASE ACTIVITY IN SERUM FROM A PATIENT WITH MYOCARDIAL INFARCTION ILLUSTRATING ELEVATION OF THE α_1 PEAK

Note small activity adsorbed at the origin. The lactic dehydrogenase activity is indicated by the solid line and malic dehydrogenase activity by the broken line.

whereas an elevation of the α_2 peak of both MDH and LDH was observed in sera from two cases of chronic lymphatic leukemia. Elevation of the α_1 peak of LDH activity in cases of myocardial infarction and of the α_2 peak of LDH activity in cases of leukemia has been reported previously (3).

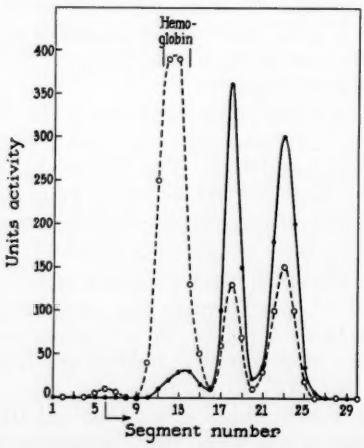


FIG. 4. DISTRIBUTION OF MALIC AND LACTIC DEHYDROGENASE ACTIVITY IN HEMOLYSATE FROM HUMAN RED CELLS

Slight activity remains adsorbed at the origin. The lactic dehydrogenase activity is indicated by the solid line and malic dehydrogenase by the broken line.

Hemolysates obtained from human red cells, separated electrophoretically and analyzed for MDH and LDH revealed activity peaks similar in mobility to those found in serum (Figure 4). The activity peaks in the fractions of the hemolysate corresponding to the α_2 and α_1 peaks of serum occurred in regions where the protein concentration was extremely low. From the point of view of purification of these dehydrogenases, it is of interest that the ratio between the activity of the enzymes and the concentration of protein in the hemolysate fractions greatly exceeds the activity protein ratio in the serum fractions.

The results of the determinations of the pH optima in each of the three peaks of the red blood cell hemolysate are shown in Figure 5. The pH optima for both MDH and LDH activity in the fraction of the hemolysate corresponding in mobility to the α_2-globulin was 8.2. The MDH and LDH activity in the hemolysate fraction corresponding in mobility to the α_1-globulin was found to be maximal at 8.5, but in the hemolysate fraction, corresponding in mobility to the β-globulin, the optimum pH for MDH differed from that for LDH. A pH of 8.9 was required for maximum MDH activity, whereas the optimum pH for LDH activity was 8.0.

Experiments with ethlyenediaminetetraacetic acid and p-chloromercuribenzoate showed that under the conditions employed, approximately 50 per cent inhibition occurred in each of the three activity peaks for both MDH and LDH.

DISCUSSION

Two of the three dehydrogenases in serum and red blood cells which reduce oxalacetate are indistinguishable in electrophoretic mobility and in pH optima from those which reduce pyruvate. A further indication of the similarity of the enzyme producing these two activity peaks is that the elevation of the α_1 peak found in serum from patients with myocardial infarction occurs with either pyruvate or oxalacetate as substrates. Similarly, elevation of the α_2 peak found in serum from patients with leukemia is present with either

substrate. Alterations in the electrophoretic conditions have failed thus far to separate the dehydrogenase activities obtained in the α_1 and α_2 peaks. In contrast, the activities in the β-globulin peak are clearly separable and thus more than one enzyme must be present.

In this connection it is pertinent that Meister has shown that many different α-keto and α-diketo acids, including oxalacetic acid, can serve as substrates for LDH obtained from rabbit muscle (7). In addition, Davies and Kun have described several α-hydroxy dicarboxylic acids which act as substrates for MDH obtained from pig heart (8). Recently, alcohol dehydrogenase from yeast has been shown to oxidize lactate and reduce pyruvate (9).

The finding of three dehydrogenases in serum which reduce pyruvate and of three which reduce oxalacetate is in harmony with recent evidence for the heterogeneity of enzymes previously thought to be homogeneous, and suggests that the elevated levels of total serum LDH and MDH found in many disease states (5, 10–12) should be evaluated in the light of this heterogeneity. The restriction of elevated levels of MDH and LDH activity to the α_2 peak in cases of leukemia and to the α_1 peak in cases of myocardial infarction, suggests that considerably more information may be obtained concerning dehydrogenase activity in disease states when fractionation is performed than when the measurement of activity is confined to whole serum.

Additional evidence for the heterogeneity of LDH is provided by Neilands, who demonstrated that crystalline LDH prepared from beef heart muscle, although monodisperse in the ultracentrifuge, exhibits two peaks of activity following electrophoresis (7, 13). Wieland and Pfleiderer have also observed the existence of more than one enzyme with LDH activity (14) in several organs of the rat. Different forms of pepsin (15), chymotrypsin (16), cytochrome c (17), ribonuclease (18), lysozyme (19), and enolase (20) also have been described.

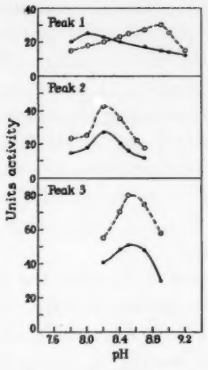


FIG. 5. CURVES SHOWING pH OPTIMA FOR THE THREE PEAKS OF ACTIVITY OBTAINED FROM RED CELL HEMOLYSATE

The lactic dehydrogenase activity is indicated by the solid line and malic dehydrogenase by the broken line.

Ultracentrifugation studies have failed thus far to demonstrate conclusively whether or not the LDH activity in serum is attributable to more than one component (21). Indeed the possibility that a single protein with polymeric forms, as in the case of muscle phosphorylase (22), might yield three electrophoretic peaks of activity cannot be entirely ruled out. Moreover, the experimental evidence presented in this paper does not eliminate the possibility of the existence of two

TABLE I

Malic dehydrogenase activity in serum fractions

	No. of cases	Total activity	Per cent total activity			Origin	α_2/α_1
			β	α_2	α_1		
Controls	10	316 (224–450)	46 (40–54)	26 (18–30)	19 (14–25)	9 (0–18)	1.4 (1.0–1.7)
Myocardial infarction	2	604	32	19	47	12	0.4
		473	28	20	41	11	0.4
Leukemia	2	233	38	49	13	0	3.7
		261	37	43	20	0	2.1

chemically distinguishable enzymes (MDH and LDH) in the α_2 peaks and of two different dehydrogenases in the α_1 peaks. However, since zone electrophoresis and pH optima have failed to reveal differentiating characteristics, the possibility that MDH and LDH activity in the α_2 peaks may be attributable to one enzyme, and that one enzyme may be responsible for MDH and LDH activity in the α_1 peaks, should not be summarily dismissed.

The suggestion (23) that the three peaks of xanthine dehydrogenase activity, which have been demonstrated electrophoretically in rat serum, are attributable to binding by lipoproteins does not apply to LDH. High speed centrifugation in a density gradient did not reveal significant activity in those fractions rich in lipoproteins.

Purified preparations of MDH and LDH isolated from tissues have been shown to possess distinguishable physical properties and substrate specificities (7, 24). However, these observations are not discordant with the results of this study, since enzymes performing similar functions in different tissues do not always have the same substrate specificities (25).

SUMMARY

1. Human serum separated electrophoretically was found to exhibit three regions of MDH activity. In 10 control subjects the percentage of MDH activity in each peak remained relatively constant, although the total activity in serum varied widely. Hemolysates from red cells separated electrophoretically revealed three peaks of dehydrogenase activity similar to those found in serum.

2. The MDH activity peaks in the α_2-globulin and between the α_1-globulin and albumin corresponded in mobility and in pH optima to the LDH activity found in the same regions. Alteration in the electrophoretic conditions thus far employed have failed to separate the proteins acting on these substrates in the two activity peaks. However, the LDH activity peak in the β-globulin differed from the MDH activity peak, both in electrophoretic mobility and pH optima.

3. The similarities between the α_2 activity peaks of MDH and LDH raise the question of whether the α_2 peaks may be due to a single protein which can act on either substrate. Similarly, the possibility that the α_1 peaks of MDH and LDH activity may also be produced by one enzyme is discussed. Further studies will be required to settle these questions.

4. It is suggested that the multiplicity of the dehydrogenase enzymes in serum should be considered in evaluating observations of total enzymatic activity in disease states.

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THE DISTRIBUTION OF ACID MUCOPOLYSACCHARIDES IN COHN FRACTIONS OF HUMAN PLASMA PROTEINS[1]

By GRACE P. KERBY

(From the Department of Medicine, Duke University School of Medicine, Durham, N. C.)

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Acid mucopolysaccharides behaving similarly to chondroitin sulfate on paper electrophoresis and paper chromatography have been demonstrated previously in human leukocytes (1, 2). A chondroitin sulfate-like substance has been recovered also from blood platelets of rats (3). A similar substance has been demonstrated in human urine (4, 5), and this substance has been identified tentatively as chondroitin sulfate A or C (6). Delay in recognizing the occurrence of chondroitin sulfate in plasma was occasioned by failure initially to recognize that the acid mucopolysaccharides of plasma appear to be in combination with plasma proteins.

Chondroitin sulfate-like substances have been recovered from human plasma now in a number of laboratories, including our own, and reports concerning recoveries and quantitative levels have appeared (7–9). The acid mucopolysaccharides are recoverable from plasma protein precipitated in the euglobulin fraction. The present report deals with a survey of Cohn fractions of plasma proteins for acid mucopolysaccharide content. Chief occurrence of acid mucopolysaccharides was noted in Fractions III and IV-1.

METHODS

Human plasma protein Cohn Fractions I, II, III, IV-1, IV-4 and V were generously furnished for the study by E. R. Squibb and Sons through the kind cooperation of Dr. J. N. Ashworth of the American National Red Cross. Duplicate 100 mg. samples were tested for acid mucopolysaccharide content by a combination and modification of the methods of Badin, Schubert, and Vouras (7) and DiFerrante (5). In brief, the method consisted of enzymatic hydrolysis and defatting[2] of the protein fraction, precipitation of acid mucopolysaccharides by the use of cetyl trimethyl ammonium bromide (5), and measurement of acid mucopolysaccharides as hexuronic acid by the carbazole method of Dische (10). The materials recovered were compared on paper chromatography (4) to commercial chondroitin sulfate (General Biochemicals, Inc.) and to heparin (Liquamin® Sodium, Organon, Inc.).

Since acid mucopolysaccharides and lipoproteins were found to occur chiefly in the same fractions, an attempt was made to determine whether the occurrence of acid mucopolysaccharides was influenced by lipid linkages. Cohn Fractions II,[3] III and IV-1 were subjected to lipid extraction by several methods: (A) ethanol-ether (3:1) in a one-phase system at 60° C. for three 10 minute periods, (B) *n*-butanol in a two-phase system with water at room temperature overnight (11), (C) *n*-butanol in a one-phase system at −15° C. (11). In each instance the lipid solvent layer was removed to tared vessels for drying and weighing of lipid residues, the residue was extracted in water which was then removed and subjected to rotary evaporation at 46° C. and measured for hexuronic acid content, and the residue itself was subjected to enzymatic hydrolysis and precipitation with cetyl trimethyl ammonium bromide and measurement of hexuronic acid content.

RESULTS

Paper chromatographic studies (Figure 1A) of material derived from 100 mg. samples of Frac-

[1] This work was supported by grants from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service, and the American Heart Association.

[2] Ethanol-precipitated protein material was finely suspended in 3 ml. of 0.1 N HCl. Crystalline pepsin (National Biochemicals Co.), 6 mg. in 1.0 ml. of 0.1 M phosphate buffer, pH 5.01, was added. Final pH was about 2.0. Digestion was visibly rapid and was continued for 90 minutes in a 37° C. water bath. Ethanol precipitation (from 80 per cent ethanol after addition of 0.18 ml. of 25 per cent sodium chloride) was repeated, and the precipitate was then extracted with 10 ml. of diethyl ether for 15 minutes, centrifuged and taken up in 4 ml. of 0.1 M pH 7.6 phosphate buffer. Crystalline trypsin (50 per cent magnesium sulfate, National Biochemicals Co.), 4 mg. in 1.0 ml. of 0.01 N HCl, was added. Final pH was about 7.0. The material was then transferred quantitatively to Visking 18/32 inch cellulose casing and dialyzed against 100 ml. of 0.1 M phosphate buffer pH 7.6 in a 125 ml. Erlenmeyer flask on a New Brunswick Gyrotory Shaker at room temperature for four hours, with one change of buffer at two hours. The material was diluted to 10 ml. with demineralized water prior to the next precipitation step.

[3] Fraction II was included as a control in all three methods.

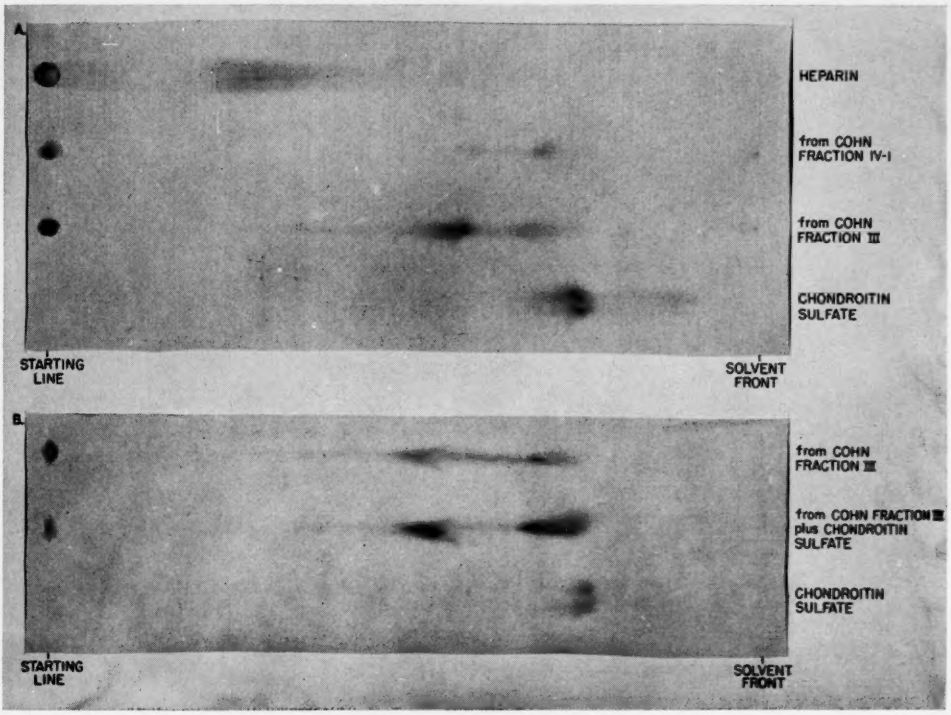

FIG. 1. FILTER PAPER CHROMATOGRAMS OF HEXURONIC ACID-CONTAINING POLYSACCHARIDES FROM COHN FRACTIONS OF HUMAN PLASMA

Solvent 48 per cent ethanol in pH 7.25 M/15 phosphate buffer.

All spots were intensely metachromatic except those at the starting line of the Cohn fraction materials, where the dark spots were predominantly insoluble material taking a bluish stain, showing no definite metachromasia in Fraction III material and only slight metachromasia in Fraction IV-1 material.

tions III and IV-1 showed two chief metachromatic components which have been seen also in studies on derivatives of the euglobulin fraction of fresh human plasma, using an ethanol-phosphate system. One component moved slightly behind the chief component of chondroitin sulfate, and the slower rate may have been due to a slight difference in the viscosity of the material inasmuch as a mixture of chondroitin sulfate with the material failed to resolve into two distinct spots in this position (Figure 1B). A second chief component moved much more slowly than chondroitin sulfate but faster than heparin; an eluate of this component showed on rechromatography the same slow spot as before as well as a small amount of the faster-moving material which resembled chondroitin sulfate; the tailing effect evident in Figure 1A and B was absent in chromatograms of eluates. A third minor metachromatic component moved with the solvent front; rechromatography of an eluate of this component revealed a single spot with mobility resembling that of chondroitin sulfate. Metachromatic material was not demonstrated in chromatograms of Fractions I and IV-4, using material derived from 100 mg. of the respective fractions. However, a concentrate derived from 400 mg. of Fraction I did reveal a small amount of definitely metachromatic material which moved slightly off the starting line in an ethanol-phosphate system, in the area of heparin mobility. It was not possible to determine whether the slow mobility was truly identical with that of heparin or whether mobility was slowed by the comparatively and considerably greater amount of extraneous material present in the large amount of material concentrated. Separation was insufficient to per-

TABLE I

Results of extraction of Cohn fractions with lipid solvents followed by aqueous extraction of the residue

Extraction procedure*	Hexuronic acid (μg. per 100 mg. of original fraction)			Weight of lipids extracted by solvent (mg. per 100 mg. of original fraction)		
	A	B	C	A	B	C
Fraction III (Total APS)		(21.8)				
Lipid solvent extract				14.5	14.8	5.3
Subsequent aqueous extract	0	0	0			
Residue, APS	22.5	†	17.8			
Fraction IV-1 (Total APS)		(16.0)				
Lipid solvent extract				22.3	20.3	19.5
Subsequent aqueous extract	5.6	0	†			
Residue, APS	11.5	15.4	10.6			
Fraction II (Total APS)		(0)				
Lipid solvent extract				0.3	†	0.1
Subsequent aqueous extract	0	†	0			
Residue, APS	0	†	0			
Lipid solvent controls				0	0.5	0.5

* A, ethanol-ether (3:1) in a one-phase system at 60° C. for three 10 minute periods; B, *n*-butanol in a two-phase system with water at room temperature overnight (11); C, *n*-butanol in a one-phase system at −15° C. (11).

† No determination possible for technical reasons. The residue of Fraction III by Method B and the evaporated aqueous phase of Fraction IV-1 by Method C presented blanks which were too dark to be visually acceptable, and no readings were made. Fraction II, which was included as a control in all three methods, could not be separated satisfactorily in the two-phase system with *n*-butanol but was carried through the other two extraction procedures satisfactorily.

mit elution of the area and rechromatography. Material derived from 400 mg. of Fraction IV-4 showed only a faint spot still slower in its mobility than the slow component of Fractions III and IV-1 and probably metachromatic; its faintness was such as to preclude photography or a more definite descriptive statement.

Hexuronic acid determinations showed no detectable color in material derived from Fractions II and V and trace amounts in Fractions I and IV-4 (equivalent to 3.9 and 10.7 μg., respectively, of hexuronic acid per 100 mg. of weighed samples of the fractions). Fractions III and IV-1 showed larger amounts (21.8 and 16.0 μg., respectively). The presence of contaminating mucoprotein or glycoprotein was shown by the relatively high hexosamine (2) values obtained on analysis of the materials derived from Fractions III and IV-1 (60.2 and 35.6 μg., respectively). The data were regarded as useful, therefore, only insofar as they offered collateral support for the observations made of metachromatic materials on paper chromatography and as they furnished a baseline for the studies involving lipid extractions.

Results of lipid extraction procedures are summarized in Table I. Extraction of Fraction III with lipid solvents failed to release demonstrable acid mucopolysaccharides to subsequent aqueous extract by any of the extraction methods used, and subsequent analysis of the residue revealed no loss of original acid mucopolysaccharide content. Extraction of Fraction IV-1 with ethanol-ether at 60° C. (Method A) was followed by demonstration of a small amount of hexuronic acid in the subsequent aqueous extract and a slight decrease in the apparent acid mucopolysaccharide content of the residue. A similar result was probably observed using *n*-butanol at − 15° C. (Method C); analysis of the aqueous extract was unsatisfactory (see Table I); the residue showed a decrease in acid mucopolysaccharide content as in Method A. The extent to which any of the methods used was successful in breaking the lipoprotein bond of the partially denatured Fractions III and IV-1 is open to doubt. The amount of lipid demonstrated in the lipid solvent extracts was less by weight than the amount which has been demonstrated (12) in lipoprotein fractions prior to drying and subsequent denaturation.

DISCUSSION

A chief site of occurrence of acid mucopolysaccharides in human plasma Cohn fractions has been shown in the present study to be those frac-

tions (III and IV-1) which contain the major portion of the plasma lipoproteins. Paper chromatographic studies of the metachromatic material recovered revealed at least two components, one of which resembled the chief component of commercial chondroitin sulfate and may possibly be the chondroitin sulfate A component described by Schiller and Dewey (8). The other component was not identified but did not resemble either heparin or components of commercial chondroitin sulfate. Both components appeared identical to those observed in the acid mucopolysaccharide-like material recoverable from the euglobulin fraction of fresh human plasma.

Attempts to break the lipid-protein bond of Fractions III and IV-1 by several procedures were only partially successful as judged by weight of lipids recovered from the lipid solvent extracts. Within the limits of the methods used and to the extent that lipid-protein bonds were severed, release of acid mucopolysaccharides for subsequent aqueous extraction could not be demonstrated from Fraction III; a small amount may have been released from Fraction IV-1. This could indicate that the hexuronate-containing polysaccharides were for the most part bound to lipids which remained bound to proteins despite the extraction methods employed. It could also indicate the possibility of binding of acid mucopolysaccharides to a protein moiety which could be lipoprotein or could be other protein fortuitously present in the fractions. The present study fails to confirm or deny an affinity between acid mucopolysaccharides and lipoproteins. It does demonstrate the possibility of a bond between the two classes of substances in that they coincide in their chief site of occurrence in plasma protein fractions.

SUMMARY

1. Cohn fractions of human plasma proteins have been investigated to determine the predominant site of occurrence of the acid mucopolysaccharides which can be demonstrated regularly in the euglobulin fraction of human plasma. By the methods employed, predominant occurrence was in Fractions III and IV-1, Fraction III showing the larger amount.

2. Paper chromatographic studies of the acid mucopolysaccharide material recovered from Fractions III and IV-1 demonstrated two chief metachromatic components. One component resembled the chief component of commercial chondroitin sulfate in its mobility. A slower component was unidentified.

3. Attempts to confirm or deny a chemical or physical binding between the hexuronate-containing polysaccharides and the lipoproteins of Fractions III and IV-1 were inconclusive.

ACKNOWLEDGMENT

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ALBUMIN METABOLISM IN CHILDREN WITH PROTEIN MALNUTRITION [1]

By DAVID GITLIN, JOAQUIN CRAVIOTO, SILVESTRE FRENK, EVANGELINA L. MONTANO, RAFAEL RAMOS GALVAN, FEDERICO GOMEZ, AND CHARLES A. JANEWAY

(From the Department of Nutrition, Hospital Infantil, Mexico, D.F., and the Department of Pediatrics, Harvard Medical School and the Children's Medical Center, Boston, Mass.)

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Of the biochemical disturbances associated with protein malnutrition in man, hypoalbuminemia is undoubtedly one of those most consistently found (1, 2). The metabolic mechanisms operating to produce this hypoalbuminemia are not known. However, analysis of diets of children with severe protein malnutrition (kwashiorkor) has revealed that the protein intake of such children is less than half of the minimal protein requirement for age, even discounting the fact that the proteins ingested are primarily of vegetable origin and that caloric intake is indaequate as well (3, 4). While it is not possible to decide *a priori* that the hypoalbuminemia in this condition is attributable to decreased synthesis secondary to diminished amino acid intake, this possibility would seem the most likely; diminished albumin synthesis has been shown to occur in animals on comparable diets (5). However, since the plasma concentration of albumin represents a balance between synthesis, distribution in the body, and degradation, any or all of these factors could play a role. Consequently, this study was undertaken to evaluate the relative influence of each of these on the production of hypoalbuminemia in children with protein malnutrition.

METHODS

Patients. Fourteen children, admitted to the Hospital Infantil, Mexico City, with severe protein malnutrition, were selected for study (Table I). All of the children had a history of prolonged inadequate protein intake and repeated episodes of diarrhea during which total food intake was greatly reduced as well. Although extremely malnourished, 3 children had no edema upon admission (Group I), while the other 11 children showed definite edema (Group II). Well-developed pellagroid skin lesions of the type seen in protein malnutrition were present in each of the 11 edematous children, but only small atrophic lesions were noted in the other three. Microscopic examination of feces revealed *G. lamblia* infestation in two children, L. B. F. and R. E. J. Patient J. M. J. died during the study, and postmortem examination revealed, among other things, the presence of bronchopneumonia.

Method of study. Each child was given five drops of Lugol's solution three times a day during the study. Within 48 hours of admission, I^{131}-labeled human serum albumin was injected intravenously, and the disappearance of radioactivity from the serum was followed for about three weeks (Period A, Table I) in all patients except J. M. J., who died eight days after the start of the study. After an additional interval of three to four weeks, eight children were given a second injection of radioiodinated albumin, and its disappearance was again followed for about three weeks (Period B, Table I). The samples of iodinated albumin used were, for the most part, prepared as described elsewhere (6), but in several instances, two preparations obtained from Abbott Laboratories were used. In each of the eight children studied on two occasions, a different iodinated albumin preparation was used for the first and second periods. The specific activities of the labeled albumins were such that a maximum of 1 mg. of iodinated albumin was injected, or no more than 1.5 microcuries per kilogram of body weight.

The condition of the children upon admission was considered to be too serious to warrant continued protein restriction. Consequently, with but one exception, during the study the patients were given either milk, later supplemented with sugar, or milk supplemented with bananas, meat, eggs and vegetables. One patient, A. V. M., was given a diet similar to that eaten before admission, namely beans and cornmeal cakes, during the first study period and then milk supplemented with meat and vegetables after this.

Three of the children, S. S. F., H. T. J. and C. R. V., were restricted to metabolic beds during the study periods for the collection of urine.

The amount of radioactivity in samples of serum or urine was determined with a well-type sodium iodide crystal scintillation counter. Proteins in the samples

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TABLE I

Albumin studies in patients with protein malnutrition

Patient	Age	Sex	Study period	Weight*	Serum* albumin	Plasma volume	Albumin in vascular system†	Albumin half-life
	yrs.			*Kg.*	*Gm. %*			*days*
Group I								
S. S. F.	3	M	A	7.10– 8.45	1.50–3.25	452	0.42	9.3
			B	9.50– 9.76	3.95–4.07	688	0.38	9.4
H. T. J.	3	M	A	5.82– 7.10	1.85–3.58	448	0.42	9.8
			B	8.30– 8.76	3.60–3.75	697	0.32	10.8
B. T. L.	5 5/12	F	A	6.80– 8.30	1.65–3.75	398	0.53	6.3
Group II								
M. X. D.	1 5/12	M	A	6.90– 8.50	1.85–4.10	373	0.54	6.6
			B	9.30– 9.30	4.30–4.30	562	0.37	7.0
J. M. J.	2	M	A	4.80– 4.50	1.55–1.87	256	0.47	7.6
L. B. F.	1 1/12	M	A	6.90– 7.60	1.50–4.10	380	0.31	10.4
			B	8.10– 8.30	3.13–3.43	498	0.27	10.8
G. M. E.	1 8/12	F	A	5.60– 6.75	2.16–3.20	401	0.31	9.0
			B	7.00– 8.00	3.75–3.75	498	0.30	10.8
C. R. V.	1 3/12	M	A	5.92– 6.70	2.62–3.05	418	0.28	8.8
			B	7.40– 8.10	3.75–4.30	468	0.32	9.5
R. E. J.	1 5/12	F	A	7.30– 7.50	2.16–3.75	455	0.34	9.3
			B	8.00– 8.25	3.43–3.43	467	0.32	8.5
A. V. M.	2 1/12	M	A	8.78– 7.85	1.92–2.35	420	0.40	14.5
			B	10.8 –11.9	4.30–4.75	561	0.30	11.3
P. B. G.	1 5/12	M	A	6.44– 5.40	0.80–1.32	518	0.22	11.0
T. H. C.	5	M	A	9.30–11.8	0.85–3.65	720	0.53	7.8
R. M. A.	6 9/12	F	A	12.0 –14.0	1.07–3.95	712	0.52	8.5
R. O. M.	3 1/12	F	A	7.50– 8.90	2.75–3.95	362	0.40	7.8

* At beginning and at end of study period.
† As fraction of total exchangeable albumin.

were precipitated in 10 per cent tricholacetic acid and the precipitates and supernates assayed for radioactivity.

Albumin concentrations in serum samples were estimated immunochemically (7).

RESULTS

The albumin half-lives in these children as determined from the serum disappearance curves are shown in Table I. The disappearance of radioiodinated albumin from the serum of some of the children is illustrated in Figures 1 to 3. There was no significant difference in the half-life of radioiodinated albumin during the second study period as compared to the first period (Table I). In the eight children who were studied twice, the half-life of albumin ranged from 6.6 to 14.5 days, with an average of 9.7 days in the first period, and from 7.0 to 11.3 days, with an average of 9.8 days in the second. The fraction of total exchangeable albumin found in the vascular compartment in these children, as estimated by extrapolation of the catabolic phase of the serum disappearance curve to zero time, is indicated in Table I. In three of the eight children studied twice, this fraction decreased appreciably from the first study period to the second (H. T. J., M. X. D., and A. V. M.); it decreased only slightly, if at all, in four children (S. S. F., J. M. J., G. M. E., and R. E. J.) and increased slightly in one child (C. R. V.). The percentage of radioiodinated albumin catabolized per day by H. T. J., S. S. F., and C. R. V., as calculated from the urinary data

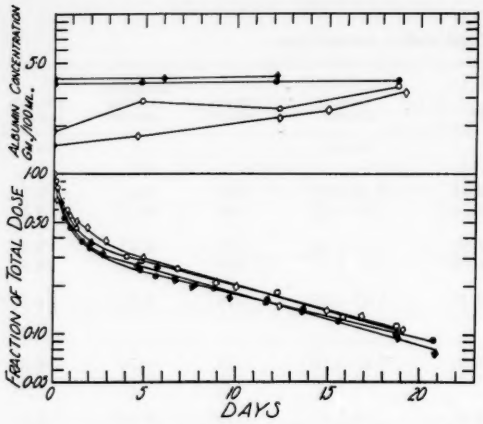

FIG. 1. SERUM ALBUMIN CONCENTRATIONS AND DISAPPEARANCE OF RADIOIODINATED ALBUMIN FROM THE SERUM IN TWO CHILDREN WITH THE NONEDEMATOUS FORM OF PROTEIN MALNUTRITION

In this and in all subsequent figures, the open symbols represent the first study period and the solid symbols indicate the second study period.

Circles, H. T. J.; diamonds, S. S. F.

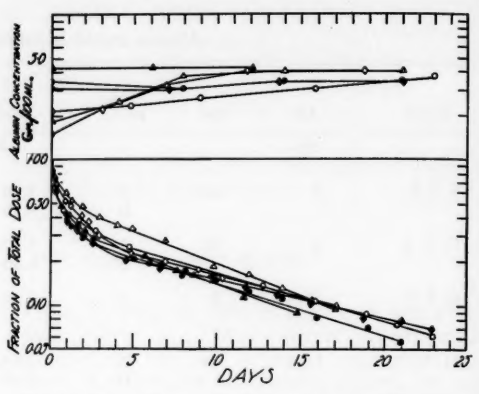

FIG. 3. THE SERUM DISAPPEARANCE OF RADIOIODINATED ALBUMIN AND THE SERUM CONCENTRATION OF ENDOGENOUS ALBUMIN IN THREE CHILDREN WITH THE EDEMATOUS FORM OF PROTEIN MALNUTRITION IN WHOM THE SERUM ALBUMIN ROSE MORE THAN 1 GM. PER CENT DURING THE FIRST STUDY PERIOD

Circles, R. E. J.; diamonds, L. B. F.; and triangles, M. X. D.

by the method of Berson, Yalow, Schreiber, and Post (8), was found to be, respectively, 6.0, 5.6 and 5.8 per cent during the first study period, and 5.8, 6.8 and 5.8 per cent during the second; these values correspond to albumin half-lives of 11.5, 12.4 and 11.9 days in the first period, and 11.9, 10.2 and 11.9 for the second. In these patients no more than 10 per cent of the administered radioactivity was excreted during the first day of the study.

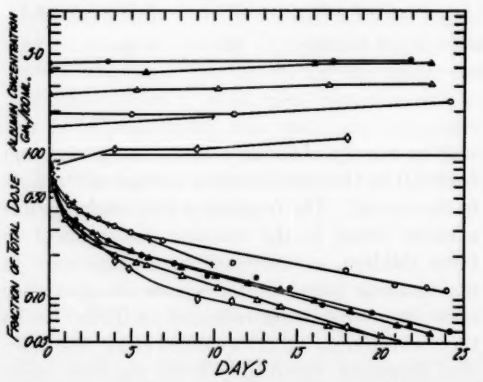

FIG. 2. THE SERUM DISAPPEARANCE OF RADIOIODINATED ALBUMIN AND THE SERUM CONCENTRATION OF ENDOGENOUS ALBUMIN IN FOUR CHILDREN WITH THE EDEMATOUS FORM OF PROTEIN MALNUTRITION IN WHOM THE SERUM ALBUMIN ROSE LESS THAN 0.52 GM. PER CENT DURING THE FIRST STUDY PERIOD

Circles, A. V. M.; diamonds, P. B. G.; triangles, C. R. V.; and crosses, J. M. J.

Serum albumin concentrations rose continuously and significantly during each first study period; the total increment in this time was from 0.32 to 0.52 Gm. per cent in four children and from 1.04 to 2.88 Gm. per cent in the others (Table I). During the second period, albumin concentrations remained rather constant, having reached relatively normal values by this time; the maximum gain in albumin during the second study period was 0.55 Gm. per cent.

DISCUSSION

Upon admission and during the study, all but one of the children were fed a diet higher in protein and calories than that which had led to their malnourished state. The effects of the diet were apparent in clinical improvement and return of serum albumin concentrations toward normal levels. Under these circumstances then, before the results of the study can be evaluated, it is necessary to decide whether the half-life of albumin deter-

mined during the initial study period truly represents the turnover of albumin in these children *before* they entered the hospital. Can it be assumed that the improved diet did not cause an immediate change in the half-life of albumin? It should be noted first that the results obtained cannot be attributed to peculiarities of an individual radioiodinated albumin preparation since several were used and in no case was the same one used for the first and second study periods. In Patients J. M. J., C. R. V., A. V. M., and P. B. G., the rise in serum albumin concentration was only 0.32 to 0.52 Gm. per cent during the study period and yet the albumin half-life in these children (Figure 2) was similar to that in the other children in whom the rise in albumin was three to six times as great during the same period of time (Figures 1 and 3). In addition, in the three of these four patients who survived, the return of serum albumin concentrations to normal occurred chiefly during the interval between the two study periods; the albumin half-life in each of the two periods was similar. And finally, although in A. V. M. the albumin half-life was similar to that in the other children, his diet during the first study period was qualitatively the same as that eaten before he entered the hospital and consisted of beans and cornmeal cakes. It would seem, therefore, that the half-lives of albumin observed initially were not drastically altered by the diet, and that the hypoalbuminemia initially observed in the children studied was attributable to some factor other than simply increased catabolism of albumin. With no loss of albumin in the urine, it may be concluded that the hypoalbuminemia was due primarily to decreased albumin synthesis.

The vascular fraction of the total exchangeable albumin in some of these children appeared to be somewhat smaller than that usually observed in normal children.[2] The urinary data suggest that this was not due to excessive albumin catabolism during the initial part of the study period in at least three of the children (H. T. J., S. S. F., and C. R. V.). In addition, the same iodoalbumin preparation that gave low vascular : total exchangeable albumin ratios in some children gave normal ratios in others. Finally, a different iodoalbumin preparation in the second study period gave low ratios in the same children in whom low ratios were obtained in the first period. In one patient, P. B. G., the serum disappearance curve (Figure 2) does suggest that albumin catabolism was faster in the initial week of study than was observed later; unfortunately, urinary excretion data in this patient were not obtained and a second study was not done. It is interesting that in at least three patients, the fraction of total albumin present in the vascular system decreased significantly from the first study period to the second. This observation is in accord with that of Yuile, Lucas, Neubecker, and Whipple, who found that protein depletion in dogs was accompanied by a decrease in extravascular plasma protein greater than that of the vascular plasma protein (9).

The average albumin half-life and the lower limits of the observed range in these children were somewhat less than those usually found in normal children.[2] The reasons for this were not apparent. Those children with an unusually rapid turnover of albumin in the first study period showed a similarly rapid albumin turnover during the second study period a month later. Whether the decreased albumin half-life could be attributed to undisclosed infection (10), or to the fact that the children had not yet fully recovered, is not known.

It is noteworthy that the metabolic response of children to protein deprivation seems to be quite different from that observed in lower animals. In mice, decreased caloric and protein intake leads to decreased albumin synthesis, but this is partially compensated by a significant increase in the half-life of albumin, reflecting a decreased rate of catabolism (5). The children observed in this study did not seem to compensate for decreased albumin synthesis in this manner.

In the three patients with the atrophic or nonedematous form of protein malnutrition, initial levels of serum albumin were lower than in many of the children in the edematous state. As has been indicated by a number of investigators, the presence of edema in this condition is not dependent solely upon the serum concentration of albumin (11, 12).

[2] In normal children the vascular compartment contains about 35 to 50 per cent of the total albumin pool and the range of half-lives for iodinated albumin is about 9 to 17 days with an average of about 12 days, depending upon the preparation used.

SUMMARY

The metabolism of serum albumin was studied in 14 children with severe protein malnutrition, using I^{131}-labeled albumin as a tracer. The half-life of albumin in these children during the hypoalbuminemic period did not change when the serum albumin concentration rose to normal values. Hypoalbuminemia in these children with low protein intakes appears to be due primarily to a decreased rate of albumin synthesis.

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TOTAL EXCHANGEABLE SODIUM, TOTAL EXCHANGEABLE POTASSIUM, AND TOTAL BODY WATER IN EDEMATOUS PATIENTS WITH CIRRHOSIS OF THE LIVER AND CONGESTIVE HEART FAILURE [1]

By L. W. BIRKENFELD,[2] J. LEIBMAN,[3] M. P. O'MEARA,[4] AND I. S. EDELMAN [5]

(From the Department of Medicine, University of California School of Medicine, and the San Francisco Hospital, San Francisco, Calif.)

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The application of isotope-dilution methods to the study of body composition has provided accurate *in vivo* estimates of the amount of body sodium, potassium, chloride, water, and fat in normal human subjects of both sexes at many age levels (1–10). Similar studies in a variety of disease states have shown changes in body composition characterized by decreases in body fat, cell mass, and body potassium, and increases in body sodium and water, when these values are expressed on a body weight basis (11–16). While this pattern of change in composition is apparent in chronically ill or acutely stressed patients who are free of edema, it is particularly pronounced in edematous subjects (13, 14, 16–18). These studies, however, have not indicated whether there are changes in the proportions of body sodium, potassium, and water which are specific for a disease state. Recently, Farber and Soberman (19) noted that edematous patients with heart disease have less body water and a higher ratio of exchangeable sodium to body water than edematous patients with hepatic or renal disease. They suggested that heart failure invokes an increase in body sodium content out of proportion to the gain of extracellular water. Similar comparative studies with respect to body potassium have not yet been reported, although low values have been noted in chronically ill patients with and without edema (15, 16, 18, 20–23). The lack of a practical method for estimating body fat, however, makes it difficult to decide whether these findings represent a loss of cell mass or a decrease in the proportion of potassium to cell mass.

These studies need to be extended because of difficulties in quantitating and interpreting changes in body composition in edematous patients. It has recently been shown that equilibrium of isotope distribution may be delayed in edematous subjects (18). The variations of body composition with sex (5, 8, 16), age (5, 7, 8, 24, 25), nutrition (5, 7, 12, 16), and treatment (15, 19, 22, 26) make it important to assure that changes ascribed to a disease are not a reflection of some of these other factors.

The objectives of the present investigation were to compare total exchangeable sodium (Na_e), total exchangeable potassium (K_e), and total body water (T.B.W.) in patients with edema due to congestive heart failure and in patients with edema due to cirrhosis of the liver, to add information on differences in body composition in edematous and edema-free patients, and to clarify the interpretation of changes in body potassium content.

Sodium^{24}, K^{42} and D_2O were used as tracers in this study, and the findings are expressed as Na_e/T.B.W., K_e/T.B.W., Na_e/K_e, Na_e/"dry" body weight, and K_e/"dry" body weight, as well as the usual expressions per unit of body weight. The results failed to confirm a disproportional increase in body sodium or a higher ratio of body sodium to body water (Na_e/T.B.W.) in patients with congestive heart failure compared with patients with cirrhosis of the liver. However, distinct differences in body composition were found

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[2] Research Fellow of the National Heart Institute, United States Public Health Service.

[3] Research Fellow of the San Francisco Heart Association.

[4] Research Fellow of the National Heart Institute, United States Public Health Service.

[5] Established Investigator of the American Heart Association.

TABLE I

Clinical data—Males

Patient number	Age *years*	Body weight *Kg.*	Transudates: Ascites	Transudates: Pleural effusion	Transudates: Peripheral edema	Remarks
			Group I. Cirrhosis of liver—edema			
2	50	67.3	+4	0	+1	icterus
3	47	80.0	+1	0	+1	icterus
6	51	41.8	+3	0	0	liver biopsy, portal cirrhosis
16	74	101.3	+4	0	+1	icterus; autopsy, portal cirrhosis
20	45	50.9	+2	0	0	esophageal varices
24	60	67.0	+3	0	0	
30	51	78.8	+3	0	0	liver biopsy, portal cirrhosis
66	48	55.5	+2	0	0	liver biopsy, portal cirrhosis
79	64	78.2	+3	0	+4	icterus
81	64	81.4	+4	0	+2	icterus
84	39	84.6	+2	0	+2	icterus
87	46	76.8	+2	0	0	icterus; hepatic coma
88	50	60.0	+2	0	+1	icterus
89	59	67.7	+3	0	+1	
91	42	64.0	+1	0	+1	icterus; liver biopsy, portal cirrhosis
92	45	60.0	+3	0	+2	icterus
Mean	52.2	69.7				
Range	39–74	50.9–101.3				
			Group II. Congestive heart failure—edema			
1	64	64.1	0	+1	+1	hypertensive and arteriosclerotic heart disease
5	64	53.9	0	+1	+1	hypertensive and arteriosclerotic heart disease
10	66	119.5	+4	0	+4	arteriosclerotic heart disease
11	76	80.0	0	0	+2	hypertensive and arteriosclerotic heart disease
12	68	59.8	0	0	+1	arteriosclerotic heart disease
18	55	41.8	0	0	+1	cor pulmonale
25	67	47.7	0	0	+1	arteriosclerotic heart disease
26	58	75.8	0	+1	+3	hypertensive and arteriosclerotic heart disease
37	79	62.3	0	0	+1	hypertensive and arteriosclerotic heart disease
47	54	48.6	0	0	+2	hypertensive cardiovascular disease
51	84	56.4	0	0	+2	arteriosclerotic heart disease
61	71	58.0	0	0	+2	arteriosclerotic heart disease
75	66	53.6	0	0	+2	heart disease of unknown type
78	40	71.4	0	0	+2	hypertensive cardiovascular disease
90	60	53.2	+3	0	+4	hypertensive cardiovascular disease
93	74	65.9	+2	0	+2	arteriosclerotic heart disease
Mean	65.4	63.3				
Range	40–84	47.7–119.5				
			Group III. Hospitalized "controls"—no edema			
4	68	86.9	0	0	0	benign gastric ulcer
9	45	69.2	0	0	0	duodenal ulcer
14	67	59.5	0	0	0	abscesses of wrist and elbow
15	72	59.3	0	0	0	cellulitis of arm and leg
31	69	48.0	0	0	0	pulmonary emphysema
34	90	43.6	0	0	0	generalized arteriosclerosis
41	73	60.5	0	0	0	cerebrovascular accident
45	74	57.0	0	0	0	anemia and purpura
49a	33	44.0	0	0	0	pulmonary tuberculosis
76	62	62.5	0	0	0	panhypopituitarism
Mean	65.3	59.1				
Range	33–90	43.6–86.9				
			Group IV. Patients with heart disease—previously edematous			
21	60	59.8	0	0	0	cor pulmonale
42	71	44.8	0	0	0	cor pulmonale
52	70	50.9	0	0	0	heart disease of unknown type
Mean	67.3	51.8				
Range	60–71	44.8–59.8				

TABLE II

Clinical data—Females

Patient number	Age *years*	Body weight *Kg.*	Transudates: Ascites	Transudates: Pleural effusion	Transudates: Peripheral edema	Remarks
			Group V. Cirrhosis of liver—edema			
19	28	39.6	+1	0	0	icterus
22	37	39.8	+1	0	0	icterus
35	43	51.8	+3	0	0	
44b	43	56.8	+3	0	0	icterus; liver biopsy, portal cirrhosis
50	39	65.7	+3	0	0	icterus; autopsy, portal cirrhosis
Mean	38	50.7				
Range	28–43	39.6–65.7				
			Group VI. Congestive heart failure—edema			
7	67	85.0	0	0	+3	arteriosclerotic heart disease
23	51	55.2	0	0	+2	cor pulmonale
49b	86	51.8	0	0	+1	hypertensive and arteriosclerotic heart disease
80	82	63.6	0	0	+1	arteriosclerotic heart disease
Mean	72	63.9				
Range	51–86	51.8–85.0				
			Group VII. Hospitalized "controls"—no edema			
40	78	41.4	0	0	0	cerebrovascular accident
39	28	36.4	0	0	0	adrenal insufficiency
72	77	43.2	0	0	0	subdural hematoma, postoperative
Mean	61	40.3				
Range	28–78	36.4–43.2				

between edematous and edema-free patients, and all patients differed significantly from normal subjects in all three categories of body composition.

SUBJECTS AND METHODS

Subjects. Fifty-seven patients were studied. They were divided into the following groups for purposes of comparison:

Group I: Sixteen edematous males with cirrhosis of the liver.

Group II: Sixteen edematous males with congestive heart failure.

Group III: Ten nonedematous male hospitalized "controls."

Group IV: Three nonedematous males with heart disease who had been edematous.

Group V: Five edematous females with cirrhosis of the liver.

Group VI: Four edematous females with congestive heart failure.

Group VII: Three nonedematous female hospitalized "controls."

Only subjects in whom an unequivocal diagnosis could be made by clinical and laboratory criteria were included for study. All patients received the customary treatment for their disease up to the week of study. The period of hospitalization, duration of treatment and loss of weight prior to the time of study were similar in the subjects with edema of cardiac and hepatic origin. The hospitalized "control" group consisted of patients without either heart disease or cirrhosis of the liver who had never been edematous. All patients were fed an analyzed diet containing 10 mEq. of sodium and 28 mEq. of potassium per 24 hours for at least one day prior to and the three days of study. Diuretics were withheld during the period of study. Food and fluid were withheld for the six hour period of D_2O equilibration.

Methods. All isotopes were administered intravenously from calibrated syringes. Three hundred μc. of K^{42} was administered in the afternoon of the first day of study. Thirty-six to 40 hours later, following a complete voiding, two consecutive spot urine specimens were collected over a three to six hour interval. Catheterization was used when necessary to ensure complete urine collection. At the time of the first spot urine collection, 45 to 100 ml. of 99.5 per cent D_2O was injected. Four and one-half and six hours later, venous blood was collected for deuterium, sodium, and potassium analyses; 150 μc. of Na^{24} was then injected. Venous samples for Na^{24} assay were collected 23 and 24 hours later.

The analytic procedures for chemical and radioactive assay have been described in detail (18). Potassium[42] was counted with an end-window Geiger-Müller tube, and Na^{24} with a well-type sodium iodide scintillation counter. Serum collected just prior to Na^{24} injection was counted as a blank correction for residual K^{42} at the time of

radiosodium assay; this correction was less than 2 per cent in all cases. D_2O concentrations were determined in triplicate on each specimen by the falling drop method. Loss of D_2O was calculated as 0.16 per cent of the injected dose per hour (18).

Calculations. Standard formulas were used in calculating specific activities, Na_e, K_e, and T.B.W. (1). Zero time was taken to be the time at which the final equilibration samples for K_e and T.B.W. were obtained. "Dry" body weight was calculated by subtracting the measured T.B.W. from the patient's body weight at zero time. Back correction of the measured Na_e to zero time was accomplished by metabolic balance from the known diet and the urinary sodium excretion during the 24 hour period of Na^{24} equilibration.

RESULTS

To avoid systematic influences on body composition because of age, sex and clinical status, the patients were grouped as listed in Tables I and II. The three primary groups for this study are the male subjects in Groups I, II and III. Tables III through VII list all the body composition data and the derived ratios of Na_e/T.B.W., Na_e/"dry" body weight, Na_e/K_e, K_e/T.B.W., and K_e/"dry" body weight. The statistical summary for the male subjects in Group I (cirrhosis of the liver with edema), Group II (congestive heart failure with edema), and Group III (hospitalized "controls") is given in Table VIII.

Total body water

The mean values for T.B.W. as per cent of body weight for edematous males with cirrhosis of the liver and congestive heart failure were 58.6 and 62.9 per cent, respectively; these values were significantly greater than the mean of 54.6 per cent for male hospitalized "controls." The difference between the mean values in congestive heart failure and in cirrhosis of the liver is not statistically significant. Although the female subjects were few in number, the values for T.B.W. in those with heart failure average less than in those with cirrhosis of the liver (See Table VII). The three males with heart disease who were no longer edematous had the highest mean value for T.B.W. as per cent of body weight (See Table VI).

Total exchangeable sodium

In the edematous male patients with cirrhosis of the liver and those with congestive heart failure, mean values for Na_e/body weight were 54.1 mEq. per Kg. and 57.8 mEq. per Kg., respectively,

TABLE III

Group I—Body composition in male patients with cirrhosis of liver and edema

Patient number	Serum Na* *mEq./L.*	Serum K* *mEq./L.*	T.B.W. *L.*	T.B.W. Body wt. *%*	"Dry" body wt.† *Kg.*	Na_e *mEq.*	Na_e Body wt. *mEq./Kg.*	Na_e "Dry" body wt. *mEq./Kg.*	Na_e T.B.W. *mEq./L.*	K_e *mEq.*	K_e Body wt. *mEq./Kg.*	K_e "Dry" body wt. *mEq./Kg.*	K_e T.B.W. *mEq./L.*	Na_e/K_e *mEq./mEq.*
2	145.0	3.07	39.26	58.3	28.0	4,152	61.7	148.3	105.8	1,785	26.5	63.8	45.5	2.33
3	144.7	3.91	43.11	53.9	36.9	3,541	44.3	96.0	82.1	3,205	40.1	86.9	74.3	1.10
6	127.7	5.18	26.86	64.3	14.9	2,417	57.8	162.2	90.0	1,438	34.4	96.5	53.5	1.68
16	139.9	3.19	50.14	49.5	51.2	5,153	50.9	100.6	102.8	2,350	23.2	45.9	46.9	2.19
20	127.1	4.87	27.66	54.3	23.2	2,359	46.3	101.7	85.3	1,665	32.7	71.8	60.2	1.42
24	133.0	3.89	37.43	55.9	29.6	3,511	52.4	118.6	93.8	2,140	31.9	72.3	57.2	1.64
30	146.1	3.55	52.28	66.3	26.5	5,386	68.3	203.2	103.0	2,819	35.8	106.4	53.9	1.91
66	150.2	4.33	32.57	58.7	22.9	3,085	55.6	134.7	94.7	1,816	32.7	79.3	55.8	1.70
79	123.2	3.54	43.53	55.7	34.7	4,300	55.0	123.9	98.8	1,680	21.5	48.4	38.6	2.56
81	143.4	3.94	52.09	64.0	29.3	5,510	67.7	188.1	105.8	2,308	28.4	78.8	44.3	2.39
84	143.4	4.07	55.55	65.7	29.0	5,470	64.7	188.6	98.5	2,775	32.8	95.7	50.0	1.97
87	125.7	3.52	40.01	52.1	36.8	2,852	37.1	77.5	71.3	2,521	32.8	68.5	63.0	1.13
88	140.2	3.78	37.92	63.2	22.1	3,284	54.7	148.6	86.6	2,197	36.6	99.4	57.9	1.49
89	135.5	3.94	35.62	52.6	32.1	3,116	46.0	97.1	87.5	1,762	26.0	54.9	49.5	1.77
91	143.3	4.27	38.33	59.9	25.7	3,308	51.7	128.7	86.3	2,415	37.7	94.0	63.0	1.37
92	134.8	4.38	38.03	63.4	22.0	3,103	51.7	141.0	81.6	2,349	39.2	106.8	61.8	1.32
Mean	137.7	3.91	40.65	58.6	29.1	3,784	54.1	134.9	92.1	2,202	32.0	79.3	54.7	1.75
S.D.‡				±5.5			±8.6	±37.5	±10.0		±5.5	±20.0	±8.9	±0.45
Range	123.2–150.2	3.07–5.18	26.86–56.12		14.9–51.2	2,359–5,510				1,665–3,205				

* mEq. per liter of serum water.

† Body weight minus total body water.

‡ S.D. $= \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$.

TABLE IV

Group II—Body composition in male patients with congestive heart failure and edema

Patient number	Serum Na* *mEq./L.*	Serum K* *mEq./L.*	T.B.W. *L.*	T.B.W. Body wt. %	"Dry" body wt.† *Kg.*	Na_e *mEq.*	Na_e Body wt. *mEq./Kg.*	Na_e "Dry" body wt. *mEq./Kg.*	Na_e T.B.W. *mEq./L.*	K_e *mEq.*	K_e Body wt. *mEq./Kg.*	K_e "Dry" body wt. *mEq./Kg.*	K_e T.B.W. *mEq./L.*	Na_e/K_e *mEq./mEq.*
1	154.5	4.55	34.05	53.1	30.0	3,122	48.7	104.1	91.7	2,454	38.3	81.8	72.1	1.27
5	141.8	4.61	33.78	62.7	20.1	3,189	59.2	158.7	94.4	1,963	36.4	97.7	58.1	1.62
10	148.0	4.74	69.64	58.3	49.9	6,983	58.4	139.9	100.3	3,293	27.6	66.0	47.3	2.12
11	146.7	4.16	39.85	49.8	40.1	3,812	47.7	95.1	95.7	2,681	33.5	66.9	67.3	1.42
12	129.2	4.88	35.00	58.5	24.8	2,359	39.4	95.1	67.4	2,599	43.5	104.7	74.3	0.91
18	146.9	4.69	24.89	59.5	16.9	1,953	46.7	115.6	78.5	1,862	44.5	110.2	74.8	1.05
25	146.2	4.13	30.91	64.8	16.8	3,113	65.3	185.3	100.7	1,665	34.9	99.1	53.9	1.87
26	137.0	6.45	49.52	65.3	26.3	5,116	67.5	194.5	103.3	2,772	36.6	105.4	56.0	1.85
37	145.1	4.65	32.41	52.0	29.9	2,971	47.7	99.4	91.7	1,986	31.9	66.4	61.3	1.50
47	137.6	4.52	31.57	65.0	17.0	3,046	62.7	179.2	96.5	1,750	36.0	102.9	55.4	1.74
51	119.5	4.74	36.78	65.2	19.6	3,385	60.0	172.7	92.0	1,424	25.2	72.7	38.7	2.38
61	122.6	4.96	39.99	68.9	18.0	3,451	59.5	191.7	86.3	2,048	35.3	113.8	51.2	1.69
75	125.3	5.88	38.87	72.5	14.7	3,659	68.3	248.9	94.1	1,699	31.7	115.6	43.7	2.15
78	139.9	3.34	50.36	70.5	21.0	3,702	51.8	176.3	73.5	3,723	52.1	177.3	73.9	0.99
90	138.5	4.25	36.34	68.3	16.9	3,733	70.2	220.9	102.7	1,571	29.5	93.0	43.2	2.38
93	144.0	3.37	47.21	71.6	18.7	4,770	72.4	255.1	101.0	1,928	29.3	103.1	40.8	2.47
Mean	138.9	4.62	39.45	62.9	23.8	3,648	57.8	164.5	91.9	2,214	35.4	98.5	57.0	1.71
S.D.				±7.0			±9.8	±49.5	±10.6		±6.9	±27.2	±12.6	±0.51
Range	122.6–154.5	3.34–6.45	24.89–69.64		14.7–49.9	1,953–6,983				1,424–3,723				

* mEq. per liter of serum water.
† Body weight minus total body water.

TABLE V

Group III—Body composition in male patients, hospitalized "controls"

Patient number	Serum Na* *mEq./L.*	Serum K* *mEq./L.*	T.B.W. *L.*	T.B.W. Body wt. %	"Dry" body wt.† *Kg.*	Na_e *mEq.*	Na_e Body wt. *mEq./Kg.*	Na_e "Dry" body wt. *mEq./Kg.*	Na_e T.B.W. *mEq./L.*	K_e *mEq.*	K_e Body wt. *mEq./Kg.*	K_e "Dry" body wt. *mEq./Kg.*	K_e T.B.W. *mEq./L.*	Na_e/K_e *mEq./mEq.*
4	146.3	4.41	43.79	50.4	43.1	3,511	40.4	81.5	80.2	3,427	39.4	79.5	78.3	1.03
9	147.7	3.94	37.81	54.6	31.4	3,040	43.9	96.8	80.4	3,126	45.2	99.6	82.7	0.97
14	146.3	4.32	30.15	50.7	29.3	2,624	44.1	89.6	87.0	2,040	34.3	69.6	67.7	1.28
15	145.1	4.59	32.34	54.5	27.0	2,646	44.6	98.0	81.8	2,112	35.6	78.2	65.3	1.25
31	145.3	5.21	27.83	58.0	20.2	2,257	47.0	111.7	81.1	2,105	43.9	104.2	75.6	1.07
34	143.6	4.37	25.21	57.8	18.4	2,287	52.5	124.3	90.7	1,491	34.2	81.0	59.1	1.53
41	132.2	4.45	29.85	49.3	30.6	2,446	40.4	79.9	81.9	1,805	29.8	59.0	60.5	1.36
45	128.7	5.10	32.12	56.4	24.9	2,707	47.5	108.7	84.3	1,996	35.0	80.2	62.1	1.36
49a	133.3	3.70	25.93	58.9	18.1	2,317	52.7	128.0	89.4	1,741	39.6	96.2	67.1	1.33
76	134.1	4.62	34.38	55.0	28.1	2,645	42.3	94.1	76.9	2,180	34.9	77.6	63.4	1.21
Mean	140.3	4.47	31.94	54.6	27.1	2,648	45.5	101.3	83.4	2,202	37.2	82.5	68.2	1.24
S.D.				±3.1			±4.1	±16.6	±4.5		±4.8	±13.9	±8.0	±0.17
Range	128.7–146.3	3.94–5.21	25.21–43.79		18.4–43.1	2,257–3,511				1,491–3,427				

* mEq. per liter of serum water.
† Body weight minus total body water.

TABLE VI

Group IV—Body composition in male patients with heart disease who were previously edematous

Patient number	Serum Na* *mEq./L.*	Serum K* *mEq./L.*	T.B.W. *L.*	T.B.W. Body wt. %	"Dry" body wt.† *Kg.*	Na_e *mEq.*	Na_e Body wt. *mEq./Kg.*	Na_e "Dry" body wt. *mEq./Kg.*	Na_e T.B.W. *mEq./L.*	K_e *mEq.*	K_e Body wt. *mEq./Kg.*	K_e "Dry" body wt. *mEq./Kg.*	K_e T.B.W. *mEq./L.*	Na_e/K_e *mEq./mEq.*
21	136.0	4.86	33.54	56.1	26.3	3,149	52.7	119.7	93.9	2,168	36.3	82.4	64.6	1.45
42	131.8	4.35	29.75	66.4	15.0	2,495	55.7	166.3	83.9	1,650	36.8	110.0	55.5	1.51
52	141.4	3.78	35.17	70.1	15.7	3,294	64.7	209.8	93.7	2,180	42.8	138.9	62.0	1.51
Mean	136.4	4.33	32.82	64.2	19.0	2,979	57.7	165.3	90.5	1,999	38.6	110.4	60.7	1.49
Range	131.8–141.4	3.78–4.86	29.75–35.17	56.1–70.1	15.0–26.3	2,495–3,294	52.7–64.7	119.7–209.8	83.9–93.9	1,650–2,180	36.3–42.8	82.4–138.9	55.5–64.6	1.45–1.51

* mEq. per liter of serum water.
† Body weight minus total body water.

both of which were significantly higher than the mean value of 45.5 mEq. per Kg. in the "control" group (Tables III, IV, V and VIII and Figure 1). These findings confirm the relationship between edema formation and increased body sodium content (18). There is, however, no statistically impressive difference in Na_e/body weight between Groups I and II (cirrhosis and heart failure) (See Table VIII).

Exchangeable sodium was expressed per unit of "dry" body weight to eliminate the contribution of edema water to weight, and the following mean values were obtained: "controls," 101.3 mEq. per Kg.; edematous male cirrhotics, 134.9 mEq. per Kg.; and edematous cardiacs, 164.5 mEq. per Kg. The values for each of the groups of edematous male patients differed significantly from those for the "controls." The Na_e/"dry" body weight was higher in the male edematous subjects with heart failure than in the edematous subjects with cirrhosis, but because of the large standard deviations, statistical significance was not achieved.

The ratio of Na_e to T.B.W. was calculated to evaluate whether there are disproportional changes in sodium content with edema formation in different diseases. The mean values for the ratio Na_e/T.B.W. were 83.4 mEq. per L. for "controls," 92.1 mEq. per L. for edematous male cirrhotics, and 91.9 mEq. per L. for edematous male cardiacs. The difference between the "control" group and the edematous groups is probably significant. There is no difference between edematous male cardiacs and cirrhotics (see Tables III, IV, V and VIII and Figure 1). This ratio is of the same magnitude in the edematous female subjects and in the nonedematous patients with heart disease as in the edematous male subjects (see Tables VI and VII).

Expressing body sodium in proportion to "dry" weight or to body water takes into account the effect of variations in absolute water content on relative body sodium content, but these ratios do not reflect the proportion of sodium to the fat-free mass of the body. The K_e, however, may be an approximate index of lean body mass even in these seriously ill subjects. The ratio of Na_e/K_e was

TABLE VII

Groups V, VI, VII—Body composition in female patients

Patient number	Serum Na* *mEq./L.*	Serum K* *mEq./L.*	T.B.W. *L.*	T.B.W. Body wt. %	"Dry" body wt.† *Kg.*	Na_e *mEq.*	Na_e Body wt. *mEq./Kg.*	Na_e "Dry" body wt. *mEq./Kg.*	Na_e T.B.W. *mEq./L.*	K_e *mEq.*	K_e Body wt. *mEq./Kg.*	K_e "Dry" body wt. *mEq./Kg.*	K_e T.B.W. *mEq./L.*	Na_e/K_e *mEq./mEq.*
						Group V. Cirrhosis of liver—edema								
19	138.6	3.68	22.69	57.3	16.9	2,228	56.3	131.8	98.2	1,203	30.4	71.2	53.0	1.85
22	140.4	4.02	22.01	55.3	17.8	1,995	50.1	112.1	90.6	1,266	31.8	71.1	57.5	1.58
35	138.2	4.41	35.52	68.6	16.3	3,772	72.8	231.4	106.2	1,525	29.4	93.6	42.9	2.47
44b	130.7	3.90	32.55	57.3	24.2	2,941	51.8	121.5	90.4	1,683	29.6	69.5	51.7	1.74
50	138.7	3.24	36.00	54.8	29.7	3,376	51.4	113.7	93.8	2,133	32.5	71.8	59.3	1.58
Mean	137.3	3.85	29.75	58.7	21.0	2,862	56.5	142.1	95.8	1,562	30.7	75.4	52.9	1.84
Range	130.7–140.4	3.68–4.41	22.01–36.00	54.8–68.6	16.3–29.7	1,995–3,376	50.1–72.8	112.1–231.4	90.4–106.2	1,203–2,133	29.4–32.5	69.5–93.6	42.9–59.3	1.58–2.47
						Group VI. Congestive heart failure—edema								
7	152.1	4.00	44.77	52.7	40.2	4,876	57.4	121.3	108.9	1,936	22.7	48.2	43.2	2.52
23	136.4	5.40	36.28	65.7	18.9	3,495	63.3	184.9	96.3	1,704	30.8	90.2	47.0	2.05
49b	140.7	2.83	30.26	58.4	21.5	3,176	61.3	147.7	105.0	1,245	24.0	57.9	41.1	2.55
80	122.2	4.88	23.99	37.7	39.0	1,957	30.7	50.2	81.6	1,164	18.3	29.8	48.5	1.68
Mean	137.9	4.28	33.82	53.6	29.9	3,376	53.2	126.0	98.0	1,512	24.0	56.5	45.0	2.20
Range	122.2–152.1	2.83–5.40	23.99–44.77	37.7–65.7	18.9–40.2	1,957–4,876	30.7–63.3	50.2–184.9	81.6–108.9	1,164–1,936	18.3–30.8	29.8–90.2	41.1–48.5	1.68–2.55
						Group VII. Hospitalized "controls"—no edema								
40	143.0	3.62	22.20	53.6	19.2	2,146	51.8	111.8	96.7	1,218	29.4	63.4	54.9	1.76
39	137.6	4.10	17.97	49.4	18.4	1,228	33.7	66.7	68.3	1,358	37.3	73.8	75.6	0.90
72	134.9	4.06	25.95	60.1	17.2	2,110	48.8	122.7	81.3	1,667	38.5	96.9	64.2	1.27
Mean	138.5	3.93	22.04	54.4	18.3	1,828	44.8	100.4	82.1	1,414	35.1	78.0	64.9	1.31
Range	134.9–143.0	3.62–4.10	17.97–25.95	49.4–60.1	17.2–19.2	1,228–2,146	33.7–51.8	66.7–122.7	68.3–96.7	1,218–1,667	29.4–38.5	63.4–96.9	54.9–75.6	0.90–1.76

* mEq. per liter of serum water.

† Body weight minus total body water.

TABLE VIII

Statistical summary of body composition in edematous male patients with cirrhosis of the liver and congestive heart failure and in hospitalized "controls"

	Cirrhosis of liver Mean ± S.D.	Congestive heart failure Mean ± S.D.	Hospitalized "controls" Mean ± S.D.	Significance of differences: Cirrhosis vs. cardiacs t*	p	Cirrhosis vs. "controls" t†	p	Cardiacs vs. "controls" t†	p
Age (range)	52.2 (39–74)	65.4 (40–84)	65.3 (33–90)						
Body weight: Kg. (range)	69.7 (50.9–101.3)	63.3 (47.7–119.5)	59.1 (43.6–86.9)						
T.B.W. % body weight	58.6 ±5.5	62.9 ±7.0	54.6 ±3.1	1.93	>0.05	2.09	>0.02 <0.05	3.49	<0.01
"Dry" body weight: Kg. (range)	29.1 (14.9–51.2)	23.8 (14.7–49.9)	27.1 (18.4–43.1)						
Na_e/body weight mEq./Kg.	54.1 ±8.6	57.8 ±9.8	45.5 ±4.1	1.13	>0.2	2.94	<0.01	3.74	<0.01
Na_e/"dry" body weight mEq./Kg.	134.9 ±37.5	164.5 ±49.5	101.3 ±16.6	1.91	>0.05	2.66	<0.02	3.87	<0.01
Na_e/T.B.W. mEq./L.	92.1 ±10.0	91.9 ±10.6	83.4 ±4.5	0.06	>0.5	2.58	<0.02	2.41	>0.02 <0.05
K_e/body weight mEq./Kg.	32.0 ±5.5	35.4 ±6.9	37.2 ±4.8	1.54	>0.1	2.45	>0.02 <0.05	0.73	>0.4
K_e/"dry" body weight mEq./Kg.	79.3 ±20.0	98.5 ±27.2	82.5 ±13.9	2.27	>0.02 <0.05	0.44	>0.5	1.71	>0.1
K_e/T.B.W. mEq./L.	54.7 ±8.9	57.0 ±12.6	68.2 ±8.0	0.60	>0.5	3.92	<0.01	2.52	<0.02
Na_e/K_e mEq./mEq.	1.75 ±0.45	1.71 ±0.51	1.24 ±0.17	0.24	>0.5	3.44	<0.01	2.81	<0.01

* $t = (\bar{x}_1 - x_2)\sqrt{\dfrac{n(n-1)}{\Sigma(\bar{x}_1 - x_1)^2 + \Sigma(\bar{x}_2 - x_2)^2}}$ (S − 3).

† $t = (\bar{x}_1 - \bar{x}_2)\sqrt{\dfrac{n_1 \cdot n_2(n_1 + n_2 - 2)}{(n_1 + n_2)[\Sigma(\bar{x}_1 - x_1)^2 + \Sigma(\bar{x}_2 - x_2)^2]}}$ (S − 3).

therefore calculated for each subject. The following mean values for the ratio of Na_e/K_e were obtained: "controls," 1.24; male patients with edema and cirrhosis, 1.75; and edematous males with heart failure, 1.71. The values in the edematous patients differed significantly from those of the "control" group, but sodium content, when referred to an approximate measure of intracellular mass (K_e), was similar in edematous subjects regardless of type of disease (see Table VIII and Figure 1). In the three subjects with heart disease without edema the Na_e/K_e ratio was intermediate between the value for the edematous subjects and the value for the hospitalized "controls" (see Tables VI and VIII).

Total exchangeable potassium

The mean values for K_e/body weight for Groups I, II, and III (cirrhotics, cardiacs, and "controls") were 32.0, 35.4, and 37.2 mEq. per Kg., respectively. Only the difference between the "controls" and the edematous cirrhotics approaches statistical significance ($0.05 > p > 0.02$) (see Table VIII). Nor are there any significant differences in K_e per Kg. of "dry" body weight between the edematous male subjects and the hospitalized "controls." The higher value in the edematous male cardiacs compared with that in the edematous male cirrhotics is of borderline significance, and the female subjects show an opposite trend (see

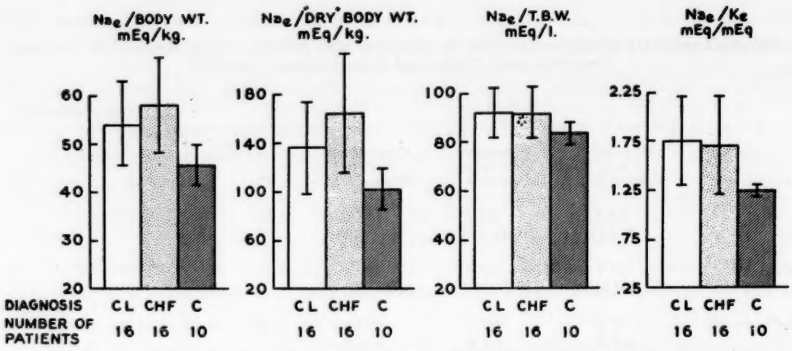

FIG. 1. COMPARISON OF Na_e IN EDEMATOUS MALE PATIENTS WITH CIRRHOSIS OF THE LIVER AND CONGESTIVE HEART FAILURE AND IN HOSPITALIZED "CONTROLS"

The mean Na_e is depicted by the height of the bar, and the length of the line is equal to twice the standard deviation. The Na_e is expressed as mEq. per Kg. of body weight, mEq. per Kg. of "dry" body weight, mEq. per L. of T.B.W., and mEq. per mEq. of K_e. CL stands for cirrhosis of the liver, CHF for congestive heart failure, and C for hospitalized "controls."

Tables VII and VIII). The values for K_e/"dry" body weight in the male subjects with heart disease but without edema also were high (see Table VI). Since the ratio of K_e to "dry" body weight reflects relative degrees of leanness as well as changes in potassium content, the latter findings probably indicate fat loss rather than conservation of potassium.

As one would expect, the ratio K_e/T.B.W. was significantly lower in patients with edema than in nonedematous subjects. The mean values for the K_e/T.B.W. ratio were 68.2 for "controls," 54.7 for edematous male cirrhotics, and 57.0 for edematous male cardiacs. The edematous groups differed significantly from the "controls," but not from each other (see Tables III, IV, V, and VIII and Figure 2). The results were the same in the female subjects (see Table VII). Indeed, these results reflect largely the diluting effect of overhydration on this ratio, and the reciprocal of this ratio (K_e/T.B.W.) is probably a fairly accurate measure of the magnitude of edema formation. The similarity of this ratio in Groups I and II probably indicates similar proportions of overhydration with respect to potassium content in these subjects.

DISCUSSION

Figures 1 and 2 depict the data on body composition in the three primary groups of patients. The Na_e/T.B.W. ratio in the edematous patients with congestive heart failure is no different from that in the edematous patients with cirrhosis of the liver. The Na_e/body weight and the Na_e/"dry" body weight in edematous cardiacs exceed the corresponding value in the patients with liver disease, but the difference is not statistically convincing ($p > 0.05$). There are, furthermore, strikingly similar differences in K_e/body weight and K_e/"dry" body weight between these two groups (see Table VIII and Figure 2). Although the higher Na_e/body weight and K_e/body weight in the cardiacs might reflect changes in electrolyte content characteristic of these disease states, it is far likelier that the patients with liver disease had a higher percentage of body fat which diluted these parameters of body composition, *i.e.*, Na_e and K_e (see Table VIII). Edema formation appears, therefore, to be associated in these groups of subjects with similar changes in body composition, regardless of the type of disease. These results do not confirm the report of a lower body water content and a higher ratio of body sodium to body water in edematous subjects with heart disease than in edematous cirrhotics (19). Exchangeable sodium per Kg. of "dry" body weight and per L. of body water is high in subjects with heart failure rendered edema free (see Table VI). The T.B.W./body weight and the ratio of K_e to "dry" body weight are higher in these three subjects than in the "controls," while K_e/T.B.W. is slightly lower (see Tables VI and VIII), which is

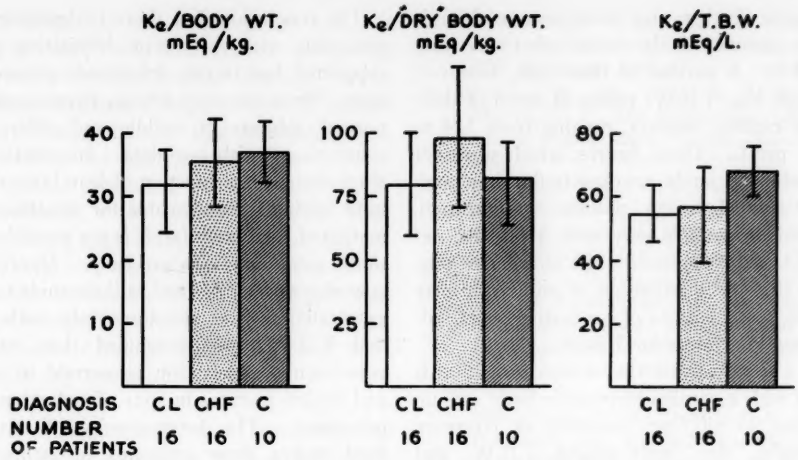

FIG. 2. COMPARISON OF K_e IN EDEMATOUS MALE PATIENTS WITH CIRRHOSIS OF THE LIVER AND CONGESTIVE HEART FAILURE AND IN HOSPITALIZED "CONTROLS"

The mean K_e is depicted by the height of the bar, and the length of the line is equal to twice the standard deviation. The K_e is expressed as mEq. per Kg. of body weight, mEq. per Kg. of "dry" body weight, and mEq. per L. of T.B.W. The diagnostic symbols are the same as in Figure 1.

consistent with the previous conclusion that greater depletion of body fat, rather than selective accumulation of sodium, is involved in the process of heart failure.

The differences in the results obtained by us and those reported by Farber and Soberman (19) deserve some analysis. The Na_e and T.B.W. data are summarized in Table IX. The results agree within 4 per cent except for the body water values and consequently the ratio of Na_e/T.B.W. in the groups with heart failure. These discrepancies could arise from differences in patient populations (*i.e.*, average fat content, influences of treatment, incidence of hyponatremia or amount of edema) or in methods of measurement. All our subjects had been treated and had long standing congestive heart failure; as a result fat content may have been lower. There is little difference in the mean and variance with respect to serum sodium concentration in the two groups [see Table IV (19) and Table IV, this paper]. While differences in the amount of edema in the two groups could account for differences in T.B.W., this would not explain the discrepancy in the Na_e/T.B.W. ratio. Our results appear to be characteristic of the patient population with heart failure at this hospital since they are in agreement with those obtained previously (18). With respect to methods of

TABLE IX

Comparison of Na_e and total body water in patients with edema

Reference	Congestive heart failure				Cirrhosis of liver			
	Subjects no.	Na_e/ body weight *mEq./Kg.*	T.B.W./ body weight %	Na_e/T.B.W. *mEq./L.*	Subjects no.	Na_e/ body weight *mEq./Kg.*	T.B.W./ body weight %	Na_e/T.B.W. *mEq./L.*
(19)	50	59.9 ±11.0	55.5 ±7.5	108.2 ±12.0	16	56.2 ±9.3	61.0 ±7.2	90.3 ±11.6
This paper	16	57.8 ±9.8	62.9 ±7.0	91.9 ±10.6	16	54.1 ±8.6	58.6 ±5.5	92.1 ±10.0
% difference		−3.6	+12.5	−16.3		−3.8	−4.0	+2.0

measurement, Farber and Soberman used antipyrine to estimate body water after checking against D_2O. A perusal of their data, however, reveals high Na_e/T.B.W. values in seven of their edematous cardiac subjects, ranging from 124 to 140 mEq. per L. These figures, which were not approached in our study, are close to the simultaneously determined serum sodium concentration. This would be possible only with a massive degree of potassium depletion. An alternative possibility is that the distribution of antipyrine may be delayed in some cases of congestive heart failure, yielding low values for T.B.W.

Figure 1 demonstrates that edema formation is associated with a parallel increase in body sodium with respect to all four standards of reference (body weight, "dry" body weight, T.B.W., and K_e), which confirms the findings in many previous studies (13, 14, 16, 18, 19, 26). Body potassium was no lower in the patients with edema than in the hospitalized "controls" when the diluting effect of edema water was eliminated. The values for K_e/body weight in these patients are in accord with those published previously (11, 15, 16, 20, 22, 23). Body potassium appeared to be abnormally low in all patient groups. The K_e/"dry" body weight in young adult males calculated from the data of Corsa, Olney, Steenburg, Ball, and Moore (2) is 129 ± 20 mEq. per Kg.[6] and from the data of Ikkos, Ljunggren, Luft, and Sjögren (9) is 137 ± 31 mEq. per Kg.[6] These figures are significantly higher than the values of 80 ± 20 mEq. per "dry" body weight [7] in the male cirrhotics and 83 ± 14 mEq. per "dry" body weight [7] in the male hospitalized "controls" (see Table VIII). The female patients all showed similar decreases in body potassium (see Table VII). The near normal values of 99 ± 27 mEq. per Kg. "dry" body weight in the edematous cardiacs and 110 ± 15 mEq. per Kg. "dry" body weight [7] in the nonedematous cardiacs probably reflect more advanced degrees of fat depletion rather than greater conservation of body potassium. Since disease is generally attended by loss of body fat, these figures relating body potassium to water-free body mass probably underestimate the degree of potassium depletion in all of these groups.

[6] Mean plus or minus one-fourth of range.

[7] Mean plus or minus standard deviation.

The conclusion that there is depletion of body potassium with chronic or debilitating disease is supported but is not definitively proved by our data. There are very few K_e figures available for normal subjects of middle and older ages for comparisons with our data. In addition, potassium content is a function of lean body mass, and until methods are available for simultaneous estimation of total body fat, it is not possible to quantitate potassium loss precisely. Moore and his coworkers (23, 27) used radiobromide to estimate extracellular fluid simultaneously with K_e, Na_e and T.B.W., and concluded that intracellular potassium concentration is normal in starvation and depletion states in spite of reductions in body potassium. The heterogeneity of extracellular fluid makes these estimates inconclusive, however, since sodium and chloride are present in cells, transcellular fluid and dense connective tissues in varying quantities which have not yet been completely defined (28–32).

A clear distinction must be made between potassium depletion and potassium deficiency. The former refers to a decrease in potassium content per unit of lean body mass which may be an obligatory consequence of the disease process, since intracellular potassium accumulation is linked with cellular energy expenditure (32). In the latter case the cells are capable of accumulating potassium when it is provided in adequate quantities. Body composition studies alone do not, of course, enable one to make this distinction.

SUMMARY

Total exchangeable sodium, total exchangeable potassium and total body water were determined in nonedematous hospitalized "controls" and in edematous patients with cirrhosis of the liver and chronic congestive heart failure with the use of Na^{24}, K^{42} and D_2O as tracers.

Total body water expressed as per cent of body weight is increased in edematous subjects; it averaged 58.6, 62.9 and 54.6 per cent in male subjects with edema and cirrhosis and with edema and heart failure, and in "controls," respectively.

Edema formation is associated with an increase in Na_e by all criteria (per unit body weight, per unit "dry" body weight, per liter of body water and per mEq. of K_e). While the Na_e/"dry" body weight was highest in the edematous cardiac

group, no difference was found in the ratios of Na_e/T.B.W. and Na_e/K_e between edematous cirrhotic and edematous cardiac subjects. Selective or disproportionate accumulation of sodium with heart disease beyond that associated with edema formation was not demonstrated.

Exchangeable potassium was decreased below normal standards when referred to either body weight or "dry" body weight in all groups of patients. There was no difference in body potassium between edematous and nonedematous subjects when referred to "dry" body weight. Edematous male cardiacs had the highest K_e/"dry" body weight, probably because total body fat was least in this group.

Limitations in interpretation of these data are discussed.

ACKNOWLEDGMENT

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STUDIES ON THROMBOCYTOSIS. I. HYPERKALEMIA DUE TO RELEASE OF POTASSIUM FROM PLATELETS DURING COAGULATION [1]

By ROBERT C. HARTMANN, JOSEPH V. AUDITORE, AND DUDLEY P. JACKSON
WITH THE TECHNICAL ASSISTANCE OF CATHERINE P. BINGHAM

(From the Departments of Medicine, Vanderbilt University School of Medicine and Hospital, Nashville, Tenn., and the Johns Hopkins University School of Medicine and Hospital, Baltimore, Md.)

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Remarkable elevation of the serum potassium concentration without associated manifestations of hyperkalemia was observed in a patient with an unexplained increase in the blood platelets (1). Studies of the phenomenon in this patient indicated that the excess of potassium was derived from the platelets during the coagulation of the blood. Hyperkalemia was encountered in certain other patients with thrombocytosis. In contrast, no striking elevation of the serum potassium concentration was observed when normal platelets were concentrated *in vitro*.

METHODS AND MATERIALS

Preparation of native blood and plasma specimens. Native (without anticoagulant) blood and plasma specimens were processed by previously described methods with reliance on the use of low temperatures and silicone-treated [2] equipment (2, 3). Platelet-free plasma was prepared from the blood specimen only after prior separation of platelet-rich plasma.

Preparation of acid-citrate-dextrose plasma. Acid-citrate-dextrose [3] (ACD) solution was added to whole blood prior to centrifugation in the proportion of one part of the anticoagulant to four parts of blood.

Isolation of platelets. Blood (300 to 500 ml.) was collected from an antecubital vein into silicone-treated tubes packed in an icebath. Saftidonor [4] sets were used for the collection of the blood in order to minimize trauma and exposure to surfaces such as occurs when blood is collected by multiple syringe technique. The blood was processed with and without the use of ACD anticoagulant. Native (without anticoagulant) and ACD platelet-rich plasmas were prepared by slow speed centrifugation (1,500 to 2,000 rpm) in a refrigerated Servall angle centrifuge. A viscid plug of platelets was isolated from platelet-rich plasma by centrifuging at higher speeds (12,000 rpm). The supernatant plasma was thoroughly removed by draining and wiping the inside of the tube carefully. The platelet plug was placed in a deep-freeze. A small red button (red cells) was noted on the bottom of the platelet plug. In order to obtain a plug free of red and white cells, the red button with a small portion of the adjacent whitish material was removed while the plug was in a frozen state. The material removed was estimated to be less than 2 per cent of the volume of the plug.

The platelet plug isolated from a measured volume of platelet-rich plasma was weighed and its volume determined by centrifugation in a graduated centrifuge tube. Platelet water was determined by allowing the wet plugs to reach constant weight by drying in an oven set at 80° C.

Blood counts. Platelet counts were performed in duplicate by the phase microscopy method of Brecher and Cronkite (4). Counts on undiluted platelet-free plasma and serum were performed as previously described (3). Red and white blood cell counts on platelet-rich and platelet-free plasma and on serum were performed in the routine manner, using appropriate pipet dilutions.

Potassium concentrations in serum, plasma and platelets. Native whole blood or plasma was placed in silicone-treated tubes for one hour at 37° C. The clots were then removed. The red blood cells in the serum from clotted whole blood were removed by high speed centrifugation in silicone-treated equipment at low temperature.

Proteins were removed from serum and plasma specimens by precipitation with 5 per cent trichloracetic acid.

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[2] Syringes and the Lusteroid centrifuge tubes were coated with General Electric Dri-film SC-87 and lubricated with General Electric Silicone Oil SF-96 (200). Other containers and apparatus were coated with Dri-film only.

[3] Acid-citrate-dextrose (ACD) solution consisted of sodium citrate, 1.32 Gm.; citric acid, 0.48 Gm.; and dextrose, 1.47 Gm. in distilled water to a total of 100 ml.

[4] Saftidonor sets were kindly provided by Dr. E. B. McQuarrie, Biochemical Research Division, Cutter Laboratories, Berkeley, Calif. The intravenous needles in these sets had been pretreated with silicone and were of No. 15 or No. 17 gauge. Only the intravenous needle and the attached, proximal 12 inches of plastic tubing were used. The distal segment of tubing and phlanged blood bottle needle were removed prior to venipuncture.

Potassium determinations were carried out in duplicate filtrates by flame photometry with a Beckman model B instrument. The variations between duplicates were as follows: 82 samples, < 0.1 mEq. per liter; 43 samples, 0.1; 19 samples, 0.2; 4 samples, 0.3; and 6 samples, 0.4. The thrombin and ACD solutions and distilled water used in these studies contained no measurable potassium.

The platelet plugs were analyzed for sodium and potassium in the following manner: Protein was precipitated with 5 per cent trichloracetic acid or digested with hot nitric acid. The trichloracetic acid precipitates were allowed to stand for 48 hours at 5° C. The suspensions were centrifuged and the supernatant analyzed for sodium and potassium by employing the Beckman DU spectrophotometer with flame attachment.

Red blood cell potassium determinations. Heparinized blood was obtained in a ratio of 1 mg. dried heparin [5] to 5.0 ml. of blood. Red blood cell potassium concentration was calculated from the whole blood and platelet-free plasma determinations, and from the average of triplicate determinations of the volume of packed red blood cells. No correction for "trapped" plasma was employed.

Case report

M. H. (Johns Hopkins Hospital No. 268267), a colored male, was first seen in 1945 at the age of 70 years with symptoms of congestive heart failure and angina pectoris. A diagnosis of hypertensive and arteriosclerotic cardiovascular disease was made. The congestive heart failure responded to therapy which included the occasional use of mercurial diuretics.

The patient's status was reevaluated in March, 1952, because of increasing congestive heart failure. Physical examination revealed grade I vascular changes of the ocular fundi. There were a few fine rales at both lung bases. The heart was enlarged to the left, and there was a soft apical systolic murmur. The pulse rate was 82 and the blood pressure was 164/80 mm. Hg. The liver was palpable 2 cm. below the right costal margin. The spleen was not palpable, and there was no lymphadenopathy. There was bilateral pitting ankle edema. Neurological examination was unremarkable.

From March, 1952, to August, 1952, the following laboratory observations were recorded. The serological tests for syphilis were negative. The volume of packed red blood cells varied between 44 and 50 per cent, and the volume of packed white blood cells and platelets between 4 and 5 per cent. The white blood cell count ranged between 9,000 and 13,000 per cu. mm. Differential white blood cell counts were repeatedly normal except for a persistent basophilia of 2 to 3 per cent. *The platelet count varied between 1,200,000 and 2,000,000 per cu. mm.* Urinalyses were normal except for occasional mild proteinuria and the finding of a rare hyaline cast. Phenosulfonthalein excretion totaled 58 per cent in two hours. *The potassium concentration in serum obtained from clotted whole blood varied between 7.0 and 8.0 mEq. per liter* when tested on five occasions and in two different laboratories. Electrocardiogram revealed no evidence of hyperkalemia. The serum sodium concentration varied between 133 and 138 mEq. per liter. The following blood chemical determinations were repeatedly normal: NPN, serum chloride, CO_2 combining power, bilirubin, cephalin flocculation, thymol turbidity, serum inorganic phosphorus, total serum proteins, albumin-globulin ratio, cholesterol, and serum alkaline phosphatase activity. Bromsulfothalein excretion test revealed 1 mg. per cent retention in 30 minutes.

Both bone marrow aspiration smears and surgical biopsy specimens revealed a marked increase in the proportion of megakaryocytes but no other abnormalities. There was no evidence of myelofibrosis or myelosclerosis.

The period from August, 1952, to the patient's death in January, 1956, was characterized by the following manifestations: increasing difficulty in control of the angina pectoris and congestive heart failure, and progressive enlargement of the liver (to the right iliac crest) and spleen (to 14 cm. below the left costal margin). There was no lymphadenopathy.

A leukocytosis developed and the platelet count and serum potassium concentration gradually fell. A typical blood examination (obtained in March, 1955) revealed the following: volume of packed red blood cells, 51.3 per cent; volume of packed white blood cells and platelets, 1.0 per cent; white blood cells, 18,350 per cu. mm.; platelets, 438,000 per cu. mm.; and serum potassium concentration, 4.8 mEq. per liter. The differential white blood cell count revealed: 1 per cent blasts, 3.5 per cent undifferentiated myelocytes, 2.0 per cent differentiated myelocytes, 6.5 per cent juvenile neutrophils, 62 per cent polymorphonuclear neutrophils, 3 per cent eosinophils, 14 per cent basophils, 6.5 per cent lymphocytes, and 1.5 per cent monocytes. Five nucleated red blood cells were noted per 200 white blood cells. At no time was anemia present.

The patient died in January, 1956. Permission for autopsy was not obtained. Although a tissue diagnosis was not possible, it was thought that the patient did represent an instance of agnogenic myeloid metaplasia.

RESULTS

A. Patient studies

Results of studies shown in Table I demonstrate that the remarkable findings of thrombocytosis and hyperkalemia noted in Patient M. H. were related. Serum obtained from clotted whole blood or platelet-rich plasma had a markedly elevated potassium concentration, *i.e.*, 7.2 to 9.6 mEq. per liter. In contrast, serum obtained from clotted *platelet-free* plasma had a normal potassium concentration, *i.e.*, 4.2 to 5.4 mEq. per liter. When plasmas with intermediate concentrations of plate-

[5] Heparin sodium, 1,000 U.S.P. units (10 mg.) per ml. was kindly supplied by the Upjohn Company, Kalamazoo, Mich.

TABLE I

The relationship between platelet levels and serum potassium concentrations

Patient and diagnosis	Platelet-rich plasma		Whole blood		Platelet-free plasma	
	Platelets ($\times 10^3$) per cu. mm.	Serum potassium mEq./L.	Platelets ($\times 10^3$) per cu. mm.	Serum potassium mEq./L.	Platelets ($\times 10^3$) per cu. mm.	Serum potassium mEq./L.
A. Patients with thrombocytosis						
M. H. Myeloid metaplasia (?)	3,120	9.6	1,210	7.2	<1	5.2
	3,470	8.8		7.8	<1	5.0
	3,220	8.0			<1	5.4
	1,610*	7.0				
	3,250	8.6			<1	4.2
	406*	4.8				
	102*	4.3				
	4,350	9.6				
R. W. Thrombocytosis, cause undetermined	2,200	7.4	1,820	6.5	1.0	4.7
R. C. Myeloid metaplasia, postsplenectomy	2,440	5.8	1,740	5.5	2.8	4.5
	2,900	6.8			<1	4.8
	2,160	6.2			<1	4.1
E. T. Myeloid metaplasia (?)	5,530	5.6	3,880	5.3	<1	4.3
M. M. Myeloid metaplasia, postsplenectomy	2,730	5.2	2,000	5.3	2.8	4.2
H. L. Chronic myeloid leukemia	1,630	5.8	978	5.4	1.7	5.1
W. B. Thrombocytosis, cause undetermined	1,210	5.6	966	5.9	<1	5.0
E. H. Chronic myeloid leukemia	1,530	4.8	1,090	5.0	<1	4.7
M. W. Myeloid metaplasia (?)	2,850	5.4	1,210	5.5	<1	5.2
B. Postsplenectomy thrombocytosis						
B. W. Idiopathic thrombocytopenic purpura	1,200	5.5	490	4.9	<1	4.8
H. M. Acquired hemolytic anemia	1,050	5.5	640	5.4	<1	5.3
N. V. Idiopathic thrombocytopenic purpura	970	5.7	541	5.3	4.0	4.8
C. Thrombocytopenic patients						
T. K. Idiopathic thrombocytopenic purpura			3.6	4.4	<1	4.3
B. W. Idiopathic thrombocytopenic purpura			4.5	4.5	<1	3.8
N. V. Idiopathic thrombocytopenic purpura			89	4.8	<1	5.2
D. Normal subjects						
S. M.	462	4.6			<1	3.9
R. H.	172	4.4	146	4.3	<1	4.5
E. N.	349	5.0	201	5.0	<1	5.3
J. G.	381	4.5	180	4.5	<1	4.6
L. C.	506	4.0	150	3.5	<1	3.6

* To obtain these levels the platelet-rich plasma was diluted with an appropriate amount of platelet-free plasma.

lets were obtained by diluting platelet-rich with platelet-free plasma prior to clotting, the resulting serum potassium concentrations were likewise intermediate in value, *i.e.*, 4.3 to 7.0 mEq. per liter. These results demonstrated that the apparent elevation of serum potassium concentration actually represented a *spurious hyperkalemia,*[6] and that the circulating *plasma* potassium concentration was normal.

Similar studies were carried out on eight other patients with thrombocytosis (Table I, Group A). In two (R. W. and R. C.) of the eight patients, the potassium concentration in serum obtained from whole blood or platelet-rich plasma was more than 6.0 mEq. per liter. In these two patients, the difference in potassium concentration in serum obtained from platelet-rich plasma and from platelet-free plasma ranged from 1.3 to 2.7 mEq. per liter. In the other six patients this difference was less than 1.3 mEq. per liter. Studies (not shown in Table I) on another patient, W. S., with agnogenic myeloid metaplasia, were of particular interest. Examination of the peripheral blood sug-

[6] The term *spurious hyperkalemia,* while possibly open to criticism from a purist point of view, was used to avoid repetition of such lengthy phrases as "hyperkalemia due to release of potassium from large numbers of platelets during coagulation." The term does not imply error in potassium determinations. Arbitrarily the term was used only when serum from clotted platelet-rich plasma had a potassium concentration more than 1.3 mEq. per liter above that of serum from platelet-free plasma.

gested megakaryocytic leukemia. An estimated platelet count was 642,000 per cu. mm. Innumerable giant abnormal platelets were noted on the smear, and there were 200 megakaryocytes and megakaryocytic fragments per 100 white blood cells. The white blood cell count was 9,900 per cu. mm. and the volume of packed white blood cells and platelets was 5 per cent. The potassium concentration in serum obtained from clotted whole blood was 10.0 mEq. per liter, but there were no associated manifestations of hyperkalemia. Following five weeks of therapy with Myleran,® the platelet count fell to 126,000 per cu. mm. There were 66 megakaryocytes and megakaryocytic fragments per 100 white blood cells. The white blood cell count was 1,800 per cu. mm. and the volume of packed white blood cells and platelets was 0.7 per cent. At this time the potassium concentration in serum obtained from clotted whole blood was 5.4 mEq. per liter. There were, therefore, four patients (M. H., R. C., R. W., and W. S.) with thrombocytosis and an associated *spurious hyperkalemia.*

Three patients with mild postsplenectomy thrombocytosis (Table I, Group B), three with thrombocytopenia (Group C), and five normal subjects (Group D) were similarly studied. In all instances the differences in the potassium concentration in serum obtained from whole blood, platelet-rich and platelet-free plasma were less than 1.0 mEq. per liter.

Results of two determinations of red blood cell potassium in one of the patients (R. C.) with *spurious hyperkalemia* were 102.8 and 105 mEq. per liter. Red blood cell potassium concentration in five normal subjects ranged between 89 and 104.8 mEq. per liter. These values agree well with those reported by Knowles, Alverson, and Rubenstein (5). Thus, the red blood cell potassium content was not strikingly increased in this patient with *spurious hyperkalemia.*

B. Special studies

Factors influencing the release of potassium from platelets

The effect of incubation of *clotted* plasma prepared from a patient (M. H.) with *spurious hyperkalemia* was studied as follows: Platelet-rich and platelet-free ACD plasma samples were clotted by the addition of thrombin solution. The clotted platelet-free plasma and a portion of the clotted platelet-rich plasma were incubated at 37° C. for one *hour* after addition of the thrombin. Thereafter, the clots were removed and the potassium concentrations were determined on the sera. The clots were removed from the other portion of platelet-rich plasma one *minute* after the addition of thrombin, and the potassium concentration was determined on the serum. Results are shown in Table II. The increase in potassium concentration in sera from platelet-rich plasma occurred within one *minute* after coagulation. These data indicate that a large amount of potassium was released rapidly from platelets into serum after coagulation had occurred. Additional incubation of clotted platelet-rich plasma at 37° C. was associated with release of still more potassium into the serum.

The effect of platelet disruption by freezing was

TABLE II

The effect of coagulation, clot retraction, and freezing on the serum potassium concentration

			Serum obtained from clotted platelet-rich plasma			Serum obtained from platelet-free plasma (platelets = <1,000 per cu. mm.)
	Platelet count of platelet-rich plasma (×10³)	Whole platelet-rich plasma	Plasma frozen and thawed five times before coagulation with thrombin*	Clot removed 60 min. after coagulation with thrombin (clot had retracted)	Clot removed one min. after coagulation with thrombin (no clot retraction)	Clot removed 60 min. after coagulation with thrombin (no clot retraction)
	per cu. mm.	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*
Patient M. H.	3,250		9.6	8.6	7.0	4.2
	3,226			8.0	7.2	5.4
Patient R. C.	2,730	7.4	7.3	5.8		4.5

* The results were essentially the same whether the platelets were removed by centrifugation prior to clotting by thrombin or not.

studied as follows: Platelet-rich and platelet-free ACD plasmas were rapidly and repeatedly frozen and thawed using an alcohol-dry-ice mixture. Some of the frozen and thawed platelet-rich plasma was centrifuged to remove platelet fragments. All of the plasma specimens were clotted by the addition of thrombin solution. After incubation for one hour at 37° C., the clots were removed and potassium concentrations determined. Results are shown in Table II. Disruption of the platelets by freezing released into the serum an additional increment of potassium (1.0 to 1.5 mEq. per liter) over and above that released by coagulation alone. The potassium concentrations in whole platelet-rich *plasma* and in *serum* obtained from frozen and thawed platelet-rich plasma were essentially identical, suggesting that freezing released virtually all of the potassium from platelets (see Table II, Patient R. C.). An aqueous suspension of platelets prepared from the blood of Patient R. C. was frozen and thawed five times and then centrifuged at high speed. The supernatant was removed and hydrochloric acid was added to the sediment in a volume equal that of the supernatant. This was allowed to stand at 5° C. for four weeks. The potassium concentration of the aqueous supernatant was 2.4 mEq. per liter and that of the hydrochloric acid used for leaching the sediment was less than 0.5 mEq. per liter. These data indicate that disruption of platelets by freezing releases most of the potassium from the platelets.

The effect of incubation of *unclotted* platelet-rich plasma prepared from Patient R. C. was studied as follows: Platelet-rich ACD plasma was incubated at 37° C. and at 2° C. At intervals, aliquots were removed and centrifuged at high speed to remove the platelets. Potassium concentrations were determined in the supernatant *platelet-free* plasma. The whole platelet-rich plasma had a potassium concentration of 6.8 mEq. per liter. Platelet-free plasma specimens, whether freshly prepared or the supernatant from incubated and centrifuged platelet-rich plasma, had a potassium concentration of 5.4 ± 0.1 mEq. per liter. It is apparent that no measurable amount of potassium was released from the platelets during incubation of unclotted plasma for 60 minutes either at 37° C. or at 2° C.

The platelets of Patient R. C. with *spurious hyperkalemia* were subjected to various experimental conditions *in vitro, e.g.,* incubation at 37° C. for several days, exposure to glass surfaces in rotating flasks, and incubation in hypotonic solutions. Survival, as measured by clot retraction (6), was not different from that of normal platelets. This patient's platelets likewise showed normal *in vivo* survival when transfused into a patient with severe thrombocytopenia. Thus there was no evidence of abnormal fragility or shortened potential lifespan.

Platelet potassium and sodium concentrations

Platelet potassium concentration was originally estimated in terms of platelet counts in plasma containing more than several million thrombocytes per cu. mm. These data suggested that the platelets in some cases of thrombocytosis might contain as much as twice the amount of potassium as normal platelets. This was not explained by a larger size of the average platelet since one of the patients with *spurious hyperkalemia* had platelets which appeared smaller than normal. Furthermore, our experience indicated that it was difficult to obtain accurate platelet counts in platelet-rich plasma containing greater than two million per cu. mm. The need for more precise measurement was obvious.

Table III shows the results of the direct determination of potassium in platelet plugs isolated from normal subjects and from patients with chronic myeloid leukemia, myeloid metaplasia and polycythemia vera. The potassium concentration of normal platelets had a mean value of 69.1 mEq. per Kg. of platelet mass (wet weight). A slight but definite increase in platelet potassium was observed in the patients with myeloid metaplasia and polycythemia vera. In chronic myeloid leukemia there was little change from the normal.

The amount of sodium in platelets was also measured. No significant difference was observed in the sodium concentration of platelets isolated from normal subjects and from thrombocythemic patients. The water content of platelets from normal subjects and thrombocythemic patients was 78.8 to 80.9 and 79.2 to 80.0 per cent, respectively. The concentrations of potassium and sodium in terms of platelet water are presented in Table III.

The volume of the packed platelet mass isolated from 40.0 ml. of normal platelet-rich plasma was 0.2 to 0.4 ml. Patients with thrombocythemia had

TABLE III

Quantitative measurement of platelet potassium and sodium

Number of experiments	Patient and diagnosis	Potassium *mEq./Kg.* platelets (wet weight)	Potassium *mEq./liter* of platelet water	Sodium *mEq./Kg.* platelets (wet weight)	Sodium *mEq./liter* of platelet water
9	Seven normal subjects	69.1 (65–71)*	86.4 (81.1–88.8)	27.0 (25–28)	33.7 (31.6–35.0)
4	Chronic myeloid leukemia (Patients B. C. and A. W.)	71.2 (67–72) $p > 0.1$†	89.0 (83.5–90) $p > 0.1$	25.0 (23–26) $p > 0.1$	31.2 (28.7–32.5) $p > 0.1$
8	Myeloid metaplasia (Patient R. C.)	74.8 (72–76) $p < 0.01$	93.4 (90–95.2) $p < 0.01$	24.5 (22–25) $p > 0.1$	30.6 (27.5–31.6) $p > 0.1$
6	Polycythemia vera (Patient C. N.)	76.1 (73–80) $p > 0.001$	96.1 (91.2–100) $p > 0.001$	25.2 (24–27) $p > 0.1$	31.5 (29.9–33.7) $p > 0.1$

* Figures in parentheses represent the range of determined values.
† p values refer to significance of the variation from the values of normal platelets.

platelet volumes of 1.0 to 3.0 ml. per 40 ml. of platelet-rich plasma. A knowledge of this volume factor plus the potassium concentration per liter of platelet mass allows one to calculate the amount of potassium contributed to platelet-rich plasma by platelets. For example, in a patient with chronic myeloid leukemia the potassium concentration of platelet-rich plasma was 8.8 mEq. per liter and that of platelet-free plasma 5.6 mEq. per liter. Forty ml. of this patient's platelet-rich plasma yielded 2.0 ml. of platelets. Hence, the volume of platelet mass per liter of plasma was 50 ml. The concentration of platelet potassium in this patient was 70.1 mEq. per liter of platelet mass (wet weight). Therefore, the amount of potassium contributed by this volume of platelets to a liter of plasma was 3.5 mEq. per liter. This figure agrees quite well with the experimentally determined value, 3.2 mEq. of potassium per liter of plasma (platelet-rich plasma K^+ minus platelet-free plasma K^+).

Influence of red blood cells on serum potassium concentration

The potassium concentration of red blood cells is approximately 25 times greater than that in plasma. Escape of potassium from the red blood cells into plasma or serum *in vitro* may significantly alter potassium levels. In the present studies, however, serum from platelet-rich plasma prepared from the patients with *spurious hyperkalemia* (M. H., R. C., and R. W., Table I) consistently had higher potassium concentrations than did serum from whole blood. During the processing of the specimens, the platelet-free plasma was actually in contact with the red cells much longer than was the platelet-rich plasma. Had the escape of potassium from the red cells been a significant factor, hyperkalemia would also be present in platelet-free plasma. Such was never the case.

Red blood cell counts in platelet-rich plasma ranged from 0 to 5,200 per cu. mm. in 16 of 18 experiments, and in two instances, the counts were 8,800 and 10,000 per cu. mm. Red blood cell counts in 14 different samples of *platelet-free* plasma were: 9 samples, 0 per cu. mm.; 4 samples, less than 20 per cu. mm.; and 1 sample, 3,400 per cu. mm.

Small amounts of normal blood (1 part to 1,000 to 5,000 parts) were added to normal ACD *platelet-free* plasma to simulate the concentrations of red cells present in platelet-rich plasma. Aliquots were frozen and thawed repeatedly with an alcohol-dry-ice mixture in order to hemolyze the red blood cells. Following clotting by thrombin and incubation of the clots at 37° C., potassium was determined on the serum. Results are shown in Table IV. An increase in serum potassium concentration occurred only after hemolysis of approximately 100,000 red blood cells per cu. mm.,

and visible hemolysis was obvious. No increase in serum potassium concentration was noted after freezing and thawing samples containing 12,500 red blood cells per cu. mm. although hemolysis was still clearly visible. Similar results were obtained in experiments performed on adjusted platelet-free plasma from two of the patients with *spurious hyperkalemia* (M. H. and R. C.). In the present studies, all plasma samples contained less than 10,000 red blood cells per cu. mm., and there was no visible hemolysis in any plasma or serum sample. These data clearly indicate that potassium escape from red blood cells did not influence the potassium determinations.

Influence of white blood cells on serum potassium concentration

Six of the nine patients with thrombocytosis had white blood cell counts greater than 10,000 per cu. mm. However, the two patients with the most marked *spurious hyperkalemia* (M. H., Table I; and W. S.) had normal white blood cell counts at the time of study. Furthermore, the only patient (H. L., Table I) with a white blood cell count more than 35,000 per cu. mm. did not have hyperkalemia. The peripheral white blood cell count in Patient R. C. (Tables I and II) ranged between 25,000 and 35,000 per cu. mm. Over two million platelets per cu. mm. were present in three samples of platelet-rich plasma prepared from Patient R. C., and the white blood cell counts on these plasmas were 2,000, 50, and 20 per cu. mm., respectively. Hyperkalemia was present in the serum obtained from these platelet-rich and white blood cell-deficient plasmas (Table II). These results suggest that the white blood cells present during coagulation did not influence the potassium determinations.

Formed elements in isolated platelet plugs

The red and white cell counts on platelet-rich plasma from which platelet plugs were isolated were in all instances less than 222 and less than 329 per cu. mm., respectively. In addition, the button of red cells and surrounding whitish material were cut away from the frozen platelet plug before cation determinations.

Influence of formed elements in serum on potassium determinations

The presence of large numbers of formed elements in *serum* could influence the potassium determinations by virtue of their content of this electrolyte. Serum obtained from clotted platelet-rich and platelet-free plasma was examined by phase microscopy in 14 experiments, including studies on three patients (M. H., R. C., and H. L.) with spontaneous thrombocytosis and one patient (N. V.) with postsplenectomy thrombocytosis. Less than 20 red blood cells per cu. mm. of serum were seen in 13 of 14 samples, and in one there were 2,000 red blood cells per cu. mm. No white blood cells were seen in the serum in 8 of 14 experiments. In the remaining six instances the white blood cell counts were: 2,900, 156, 150, 100, 50, and 6 white blood cells per cu. mm. of serum. No platelets were seen in the serum in 11 of 14 experiments. In three instances the platelet counts were: 4,000, 3,000, and 2 platelets per cu. mm. of serum. Thus, no formed elements were present in the serum prepared from most samples of platelet-rich and platelet-free plasmas. The few

TABLE IV

Effect of red blood cells on serum potassium concentration

Red cells per *cu. mm.*	112,000	12,500	5,800	1,096	3
White cells per *cu. mm.*	203	20	10	2	0
Platelets per *cu. mm.*	3,700	594	286	128	161
I. Untreated plasma					
Serum potassium (*mEq./L.*)	3.6	3.5	3.5	3.5	3.6
Appearance of serum	Not hemolyzed	Not hemolyzed	Not hemolyzed	Not hemolyzed	Not hemolyzed
II. Plasma frozen and thawed					
Serum potassium (*mEq./L.*)	4.4	3.5	3.5	3.5	3.5
Appearance of serum	Cherry red	Slightly red	Slightly pink	Not hemolyzed	Not hemolyzed

formed elements seen in occasional specimens were insufficient to influence the serum potassium determinations. Serum from clotted whole blood contained many formed elements, particularly red blood cells. These, however, were removed from the serum by high speed centrifugation prior to the potassium determinations.

DISCUSSION

The studies presented clearly indicate that some patients with thrombocytosis may have an associated *spurious hyperkalemia* in the absence of symptoms or signs of hyperkalemia. The circulating plasma in these patients contained a normal potassium concentration, as demonstrated by the fact that sera prepared from platelet-free plasmas had normal potassium concentrations. Initially, the etiology of the thrombocytosis in the first patient (M. H.) studied was obscure. Subsequently the patient developed the picture of agnogenic myeloid metaplasia. The thrombocytosis was associated with agnogenic myeloid metaplasia in three of the four patients with *spurious hyperkalemia,* and the fourth patient had thrombocytosis of undetermined etiology. The elevated potassium concentration in serum prepared from clotted blood returned to normal in one patient (W. S.) with myeloid metaplasia when the platelets were suppressed by therapy with Myleran.® A similar observation has been reported following therapy with radioactive phosphorus (7).

In any patient with an unexplained hyperkalemia, platelet concentrations should be measured by platelet count or determination of the volume of packed platelets. If the platelets are elevated, potassium concentration should be measured in serum obtained from carefully prepared platelet-free plasma. Others (7) have reported elevated potassium levels in platelet-free as well as platelet-rich plasma from similar patients, but it is not clear that the methods employed were adequate to prevent escape of potassium from the platelets and red blood cells during the preparation of platelet-free plasma.

Potassium was released from the platelets during or after the process of coagulation. It was, however, not necessary for the clot to undergo retraction in order to bring about release of potassium from the platelets. The potassium concentration was markedly elevated in serum from platelet-rich plasma within one minute after coagulation by thrombin (Table II). Previous studies have suggested that platelets undergo profound changes within seconds after coagulation occurs using similar experimental conditions (6). Clot retraction was associated with a further release of potassium into the serum (Table II). Whether this was due to clot retraction *per se* or simply to the additional period of incubation required for retraction to occur is not known.

Vasoconstrictor (serotonin) activity has been reported to be low in serum obtained from some patients with thrombocytosis (8, 9). The biological assay used is based on the degree of constriction of vascular and other muscle tissue suspended in the serum. The potassium concentration in fluid media is known to influence muscular activity. The *spurious hyperkalemia* present in some cases of thrombocytosis might influence serum vasoconstrictor activity determinations.

The method employed in isolating platelet plugs provides a means of measuring platelet cations under the most physiological conditions possible. The isolation of platelets from blood in the absence of an anticoagulant was necessary, since use of the anticoagulant, ACD, was found to reduce platelet potassium by as much as 5 to 10 per cent. A similar loss of potassium has been demonstrated in red cells collected in ACD solution (10).

When platelet potassium was estimated on the basis of platelet counts, the data suggested that the concentration of this cation in thrombocythemic platelets might be much greater than that in normal platelets. On the other hand, direct determination of potassium in isolated platelets showed only a relatively slight increase above normal in patients with thrombocytosis (Table III). This increase could not account for the abnormally high serum potassium observed in such patients. It is concluded from these studies that the *spurious hyperkalemia* was mainly due to an increased platelet mass per unit of blood or plasma, and only slightly to increased platelet potassium concentration.

The initial misleading estimates based on platelet counts were apparently due to difficulty in counting all of the platelets in thrombocythemic plasma. When such large numbers of platelets are concentrated in platelet-rich plasma during centrifugation, there appears to be great likelihood that these elements will collide and form clumps.

Under such circumstances platelet counts may greatly underestimate the number of platelets present. In patients with thrombocytosis, it appears essential to employ platelet volume or weight studies in order to ascertain platelet mass.

The sodium, potassium and water contents of platelets observed in the present study are interesting. The water content (80 per cent) was found to be similar to that of the white blood cell (11) but different from that of the red blood cell (65 per cent). The concentrations of the cations, sodium and potassium are only slightly different than in the red blood cell. The present study demonstrates that the cation composition of human platelets shows the pattern of intracellular fluid, *i.e.*, a preponderance of potassium and a relatively small amount of sodium. These findings suggest that platelets may be enveloped by a semipermeable membrane, and that they may possess a sodium and potassium pump mechanism. Experiments are in progress to test the latter hypothesis.

SUMMARY

Elevated serum potassium concentrations were noted in 4 of 13 patients with thrombocytosis in the absence of symptoms or signs of hyperkalemia. This represented a *spurious hyperkalemia* due to release of potassium from the platelets during coagulation, since serum prepared from platelet-free plasma had a normal potassium concentration.

Coagulation, incubation of clotted plasma, and disruption of platelets by freezing were associated with release of additional increments of potassium from the platelets. Potassium was released from the platelets immediately after coagulation and prior to retraction of the clots. Potassium was not released from the platelets during incubation of unclotted plasma for a period of one hour.

The mean potassium concentration of normal platelets was found to be 69.1 mEq. per Kg. of platelets (wet weight) or 86.4 mEq. per liter of platelet water. There was no appreciable increase in platelet potassium in chronic myeloid leukemia. Platelet potassium was relatively increased in patients with polycythemia vera and myeloid metaplasia. This increase, however, was insufficient to account for the *spurious hyperkalemia* present in some patients with thrombocytosis. It was concluded that the hyperkalemia was mainly due to an increased platelet mass per unit volume of blood or plasma, and only slightly due to increased platelet potassium concentration.

Platelets were found to have a water content of approximately 80 per cent and to contain small amounts [25 mEq. per Kg. of platelets (wet weight)] of sodium. The method employed provides a means of measuring platelet cations under the most physiological conditions possible.

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CHARACTERIZATION OF THE PROTEINS OF CERTAIN POST-MORTEM HUMAN SYNOVIAL FLUIDS[1, 2]

By K. SCHMID AND MARGARET B. MACNAIR

(From the Department of Medicine, Harvard Medical School, and the Medical Services of the Massachusetts General Hospital, Boston, Mass.)

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In a recent publication (1), the first part of our study on the characterization of the synovial fluid proteins, evidence was presented that the protein components of pathological joint fluid are identical with those of blood plasma. In the present communication the proteins of postmortem synovial fluid from patients who had no signs or symptoms of joint disease are described.

This study was carried out in three sections. First, postmortem synovial fluid was analyzed electrophoretically. Secondly, immunoelectrophoresis was performed to gain information concerning the nature of the proteins of postmortem joint fluid. Thirdly, to quantitate the latter results the proteins were separated into six solubility groups by the low temperature-low salt-ethanol procedure, Fractions I–VI, and further characterized.

MATERIAL AND METHODS

The synovial fluid specimens were aspirated within 5 to 10 minutes after death from knee joints of patients clinically free of joint disease. The specimens were collected without anticoagulant; they appeared clear, *i.e.*, no clot was observed. They were kept at − 30° C. until enough material had been obtained to start investigation. The volume, protein concentration and cholesterol content of individual and pooled samples were measured (1). No attempt was made to determine the exact degree of edema, although the largest volumes of fluids were obtained from patients with probable edema.

To render possible the characterization on the synovial fluid proteins, prior to analysis all joint fluid samples were incubated at 37° C. for a two hour period with 0.2 ml. of bovine testicular hyaluronidase solution per 1 ml. of fluid (3.0 mg. of enzyme dissolved in 1.2 ml. of 0.15 M NaCl solution; the enzyme preparation contained 1,050 turbidity reducing units per mg.) (1, 2). For the extremely viscous samples,[3] designated as gel-like in Tables I and II, the incubation time had to be extended to three hours because the viscosity was reduced at a much lower rate. As shown earlier (2), hyaluronidase does not alter the plasma or synovial fluid proteins and does not influence their analyses.

Immunoelectrophoresis has recently been described in detail (1, 5–7). The synovial fluid specimens used for the present analysis and listed in Table I were digested separately with hyaluronidase, combined and lyophilized. The residue was dissolved in 2.5 ml. of distilled water, dialyzed against water at 2° C. for 8 hours to remove most of the inorganic salt and further dialyzed against pH 8.6, Γ/2 0.07 borate buffer for 17 hours. The resulting solution which had a volume of 4.0 ml. and a protein concentration of 7.5 per cent was then employed for immunoelectrophoresis.

The procedure for the fractionation of the proteins, based on the low temperature-low salt-ethanol method in conjunction with the use of heavy metal ions, has also been described in detail (2). The joint fluid samples employed for these investigations had been aspirated from 40 knee joints and were digested individually with a total of 17 mg. of hyaluronidase dissolved in 15 ml. of 0.15 M NaCl solution. They were pooled with a resulting volume of 114 ml. corresponding to 2.4 ml. per knee joint. This series showed a large variation in the individual fluid volumes (0.5 to 6.0 ml. per joint) and in the color (from almost colorless to dark brown). Most of the samples had a high viscosity as judged by their gel-like appearance. Two samples were of medium viscosity and only one exhibited a low viscosity. For the fractionation of the proteins 80 ml. of the digested fluid was used. The methods employed for the subsequent characterization of the synovial fluid proteins were the same as those applied previously (1). Normal human plasma was fractionated simultaneously to make possible a comparison of the proteins of these two body fluids.

RESULTS

I. *General observations*

The frozen synovial fluid specimens appeared turbid when thawed and, following enzymatic di-

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[3] The viscosity of the postmortem synovial fluid specimens was estimated roughly. The exact viscosity of such fluids had been described earlier (3, 4).

TABLE I

Synovial fluid specimens used for immunoelectrophoresis *

Case no.	Age *yrs.*	Diagnosis	Volume of fluid from one knee† *ml.*	Fluid taken
1	82	Myocardial infarct	0.9	Premortem
2	70	Cancer of lung	1.2	Postmortem
3	81	Thrombosis	1.2‡	Premortem
4	58	Myocardial infarct	0.2	Two mins. after death
5	68	Multiple cerebral vascular accident	1.0	Premortem
6	65	Septicemia	1.5	Premortem

* All fluids were straw-yellow and of medium viscosity.
† Although the fluid from both knees was used for this experiment, the average volume of the fluid from both knees is given here in order to compare these values with those reported by others (3, 4).
‡ This sample included the fluid from one elbow joint.

gestion, yielded insoluble material. The joint fluid samples listed in Table II were divided in six groups arranged according to increasing estimated viscosity. It appears that with decrease of fluid volume, there was an increase in protein concentration ranging from 0.9 to 2.9 per cent. A similar observation was made with respect to the cholesterol content inasmuch as the latter value increased with increasing protein content as judged by the pooled samples. The cholesterol content of the specimens listed in Table II, which was found to be significantly lower than that of pathological or traumatic joint fluid (1), varied from 5 to 14 mg. per 100 ml. of fluid. In addition, it should be noted that the fluid samples listed in Table II showed a relatively constant cholesterol-protein ration of only 6 mg. per gram as compared with a value of 23 to 32 mg. per gram of normal human plasma proteins.

For comparative purposes the cholesterol content of pooled bovine synovial fluid which was taken from the astrogalotibial joints of young steers [4] immediately after their death was also studied. The protein concentration was 1.2 per cent, and the cholesterol content was found to be 16 mg. per 100 ml., or 13 mg. cholesterol per gram of protein.

II. *Specific results*

A. Electrophoretic investigation. The electrophoretic distribution of the proteins of postmortem synovial fluid, as observed under standard conditions (pH 8.6, $\Gamma/2$ 0.1 diethyl-barbiturate buffer), indicated that of proteins present, the relative proportion of albumin was higher in joint fluid than in normal plasma (Table II). The relative increase was most pronounced with fluids of patients from whose joints only a small volume of fluid could be aspirated (Group 6, Table II). Furthermore, the presence of α_1- and α_2-globulins as well as the other electrophoretic groups of globulins corresponding to those of plasma were observed consistently (8, 9). The relative concentration of these proteins could easily be determined since the analyses were performed on specimens which had been digested with hyaluronidase (Tables II and III). In undigested specimens the high viscosity produced by hyaluronic acid blurred the electrophoretic patterns sufficiently so as to make quantitative measurements difficult.

Typical of all fluids reported in Tables II and III was the relatively low concentration of the α_2-globulins which was consistently lower than that of the α_1-globulins (1, 10) in contrast to the distribution of the proteins of normal plasma. The electrophoretic albumin-globulin ratio ranged from 1.4 to 2.1, as compared with a value of 1.2 for normal human plasma. Electrophoresis at pH 4.5 suggested the presence of orosomucoid (11, 12) and certain α_2-glycoproteins distinguished by their low molecular weights (13).

B. Immunoelectrophoretic investigation. The average protein concentration of the synovial fluid specimens employed for the immunoelectrophoresis was 2.7 per cent and the average volume 1.0 ml. per knee joint (Table I). The immunoelec-

[4] These animals had been used for a study on growth at the Iowa State College for Agriculture and Mechanic Arts, Ames, Iowa.

TABLE II

Electrophoretic study of postmortem synovial fluid *

Group	Case	Age *yrs.*	Diagnosis	Volume of fluid from one knee† *ml.*	Color of the fluid	Rough estimate of the viscosity of the fluid	Protein concentr. (Biuret) %	Cholesterol content *mg./ml. fluid*	Ratio of cholesterol: protein *mg./Gm.*	Electrophoresis at pH 8.6 (relative percentage) Alb.	α_1	α_2	β_1	β_2	γ	Protein conc. %	A/G ratio (electrophoretic)	Electrophoresis at pH 4.5 (relative percentage) "α_1"	"α_2"	Rest
1	Av	74	Carcinoma of the prostate, metastases	3.7	Colorless	Low	1.0	0.04‡		61	8	5	11	4	11		1.6	3	4	93
2	Da	48	Hepatic coma (alcoholic)	5.4	Yellow	Medium	0.9	0.03‡		62	4	2	9	4	19	1.4	1.6	1	4	95
3	Yo	70	Myocardial infarct	3.9	Light yellow	Medium	1.0	0.05	5	62	6	5	15	3	9	1.5	1.6	4	3	93
4	Lo	37	Hypertension	1.9	Colorless															
	Co	75	Broncho-pneumonia	0.8	Light yellow															
	Ba	17	Septicemia	1.6	Bright yellow	Medium	1.4	0.09	6	58	10	5	11	3	13	1.8	1.4	5§	4	91
	Si		Cerebral vascular accident (C.V.A.)	1.4	Colorless															
5	Mc	48	Hepatic coma, cirrhosis	3.0‖	Yellow	Almost gel-like	0.9			62	6	5	15	3	9	0.7	1.6			
6	Le	85	Carotid occlusion	1.7 ¶																
	Wa	66	C.V.A.	0.2	Yellow	All gel-like	2.9	0.14	5	68	4	2	13	4	9	1.7	2.1	2	4	94
	Fr	52	C.V.A.	0.6																
	Fo	50	C.V.A.	0.3 ¶																
	Mw	55	Uremia	0.2																

* All values are corrected for the dilution due to the addition of hyaluronidase solution.

† Again the average fluid volume of both knees is quoted, although for the experiments the total fluid is used. Some of the fluids from different patients were pooled as indicated by the brackets.

‡ Reading on Coleman below 0.09.

§ After electrophoresis at pH 8.6, combined and rerun at pH 4.5.

‖ Contained bilirubin and was taken 3.5 hours after death.

¶ Taken premortem.

TABLE III

Fractionation of pooled postmortem synovial fluid from patients clinically free of joint disease *

Fraction	Total protein† mg.	Total hexose mg.	Total hexose %	Total cholesterol mg.	Electrophoresis at pH 8.6 (expressed in relative percentage) Alb.	α_1	α_2	β_1	β_2 Fibr.	γ	Electrophoresis at pH 4.5 (relative percentage)	$S_{20,w}$‡	Total albumin 'HBABA'§ mg.	Total β_1-metal combining protein mg. %	Total cerulo-plasmin mg.	Total anti-strepto-lysin-O units	Total diph-theria anti-toxin units	Titer of isoagglutinins
80 ml. pooled postmortem synovial fluid	1,200(1.5%)			19	57	8	4	18	3	10	'α1' 4; 'α2' 4; Rest 92		880			960–1,280	24–80	anti-A 1:8; anti-B 1:4
I	69‖	0.6	1.7									8.2; 6.6						
II	0.1 } 430																	
III	430 }	13.4	3.1	15	11	7	15	32		35		14; 6.8			15	1,280	8–40	anti-A 1:16; anti-B 1:16
IV	272 } 722	5.9	2.2	2.8	83	11	2	4				4.6	230	10				
V	450 }	5.8	1.2		91	4	2	3				4.3 ¶	253					
VI	110	8.6	7.8		27	46	8	18	1		'α1' 53; 'α2' 6; Rest 41	3.2 ¶,**						
20 ml. acid-citrate-dextrose—normal plasma	1,400(7.0%)			36	53	5	10	15	7	10			680					
I	70	2.0	2.8		8		2	5	79	6		16; 6.4						
II	170 } 404	3.2	1.9		3	2		8	5	82		6.8 ¶						
III	234 }	8.7	3.7	22	3	8	41	31	9	8		13.2; 7.0			14			
IV	88 } 716	3.3	3.7	6	49	21	15	12	3			4.6		1.0				
V	628 }	2.5	0.4		93	2	1	4				4.1 ¶	390	18				
VI	22	2.0	9.1		38	31	15	12	4		'α1' 42; 'α2' 5; Rest 53	3.3 ¶						

* Obtained from patients with various diseases. The relatively large volume of some of the joint fluids indicated varying degree of edema. For the determination of the biological activities an aliquot of Fraction III was dialyzed at 2° C. against 0.15 M NaCl to remove the ethanol. The insoluble material was then centrifuged off and 1 ml. of the supernatant solution adjusted to the original fluid volume was used for each test. Electrophoresis of Fraction VI at pH 8.6 and 4.5 was carried out on paper.

† Biuret.

‡ In 0.15 M NaCl at a protein concentration of approximately 1 per cent.

§ 'HBABA' refers to determination of albumin by the "2-(4'-hydroxy-benzene-azo)-benzoic acid" method (22).

‖ Includes 22 mg. of insoluble material obtained prior to fractionation.

¶ Sedimentation constant of heavier component not indicated.

** A component with smaller sedimentation constant was observed.

trophoretic analysis revealed the following results: The number, intensity and relative position of the precipitin lines which were essentially the same for both synovial fluid and normal human plasma suggested that most of the proteins of postmortem synovial fluid are, with respect to many properties, very similar to those of plasma. This similarity of the γ-globulins of joint fluid to those of plasma was further proven by the closing of the corresponding precipitin zones, which is indicated in Figure 1 by the arrow on the left. The blood serum analysed simultaneously with the synovial fluid showed an additional unidentified component in the β_2-globulin region. Most of the postmortem as well as the traumatic synovial fluids showed a prealbumin component which is probably identical with that of serum (14, 15).

This method, of course, would only identify proteins present in plasma and any proteins not present in plasma would remain undetectable. However, if an antiserum against normal human joint fluid is used in future studies, possible components produced solely by the synovial tissue might be detected.

C. Fractionation and characterization of the proteins of postmortem synovial fluid. The protein concentration of the pooled joint fluid used for these experiments (Table III) was 1.5 per cent and averaged 1.7 per cent with respect to the undigested fluids. This finding suggests again

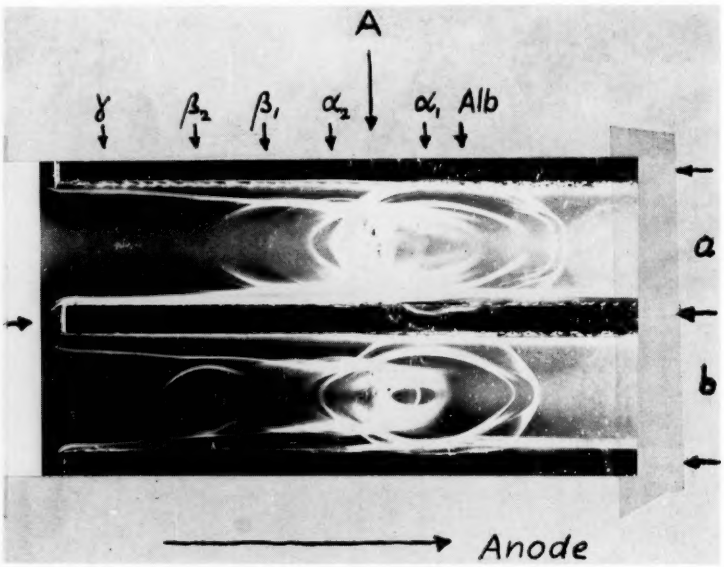

FIG. 1. IMMUNOELECTROPHORESIS OF POSTMORTEM HUMAN SYNOVIAL FLUID (a) AND NORMAL HUMAN SERUM (b)

The pooled postmortem joint fluid specimens (Table I) were treated with hyaluronidase prior to electrophoresis. "A" points to the position of application. Electrophoresis was carried out in borate buffer, pH 8.6, $\Gamma/2$ 0.07, containing 2 per cent agar, for 17 hours at 180 V. and 40 ma. The arrow on the bottom indicates the direction of the electrophoretic movement of the proteins towards the anode. After electrophoresis the troughs between the agar sections indicated by arrows on the right were filled with horse serum against human serum. Therefore, the precipitin zones formed symmetrical patterns. According to this analysis the joint fluid is devoid of a strongly antigenic protein of the β_2-globulins which is clearly visible in the picture of the serum. The γ-globulins of synovial fluid and normal serum produced precipitin lines which formed a closed system (not visible), the position of which is indicated by the arrow on the left.

that some of the specimens had been obtained from patients with edema. The ultraviolet absorption curve showing a maximum at 280 mμ and a minimum at 255 mμ was characteristic of proteins. There was no evidence of nucleic acid in the absorption spectrum. Electrophoretic analysis indicated a relative albumin content of 57 per cent and an albumin-globulin ratio of 1.3. The cholesterol content was 0.24 mg. per ml. or 16 mg. per gram of protein. The discrepancy between the cholesterol content of this series and that of the specimens used for the electrophoretic investigation can perhaps be explained by a relatively increased permeability of the lipoproteins through the synovial tissue due to edema. Further studies on normal synovial fluid are required to corroborate these findings.

The insoluble material of the pooled fluids obtained after enzymatic digestion and unavoidable repeated freezing was centrifuged and washed. It amounted to 34 mg. or 2 per cent with respect to the total amount of protein and contained 5.4 per cent of protein-bound hexose as determined by the orcinol method of Sørensen and Haugaard (16), 1.0 per cent of glucosamine measured by the method of Rimington (17), and less than 0.2 per cent of uronic acid determined by the method of Fishman and co-workers (18). It was concluded that this material did not contain significant amounts of hyaluronic acid breakdown products and represented, at least in part, denatured protein.

As judged by the characteristics of the protein fractions [the distribution of the cholesterol between Fractions III and IV; amount, purity and electrophoretic mobility of the γ-globulin in Fraction II; low content of albumin in Fraction IV and high purity of the albumin in Fraction V (1, 2)] derived from normal human plasma that had been fractionated simultaneously with the synovial fluid, it must be concluded that the deviations observed with these synovial fluid protein fractions (γ-globulins not extractable as Fraction II, relatively large amount of Fraction VI) did not stem from the fractionation process, but were inherent in the synovial fluid itself.

The characterization of the synovial fluid proteins based upon the investigations of the six protein fractions revealed the following results: The distribution of the proteins among the fractions was similar to that of normal human plasma. The hexose content of the corresponding fractions was the same within the error of the method. The hexose content of Fraction V was low and that of Fraction VI high as found previously in the corresponding fractions of pathological fluids (1).

Fraction I dissolved readily at room temperature in the appropriate salt solution (1) and remained clear for at least 14 hours, *i.e.*, no clot was formed. Upon prolonged cooling (17 hours) at 2° C. insoluble material separated which dissolved easily when the suspension was warmed to room temperature. This solubility property is characteristic of the so-called "cold insoluble" globulin (19). The γ-globulins which usually represent the major component of *Fraction II* could not be extracted from Fraction II plus III probably because of the presence of only a relatively small amount of β_1-lipoprotein which, in addition, had perhaps been partially denatured by the process of repeated freezing and thawing. Indeed, the γ-globulins remained in *Fraction III* as judged by electrophoretic analysis and from the results of the determinations of the antistreptolysin-O and diphtheria antitoxin content. The antistreptolysin-O content amounted to 12 to 16 units per ml. of the digested pooled synovial fluid, a value which, after correction for the protein concentration, is essentially the same as that found for pooled, normal human plasma (76 to 80 units per ml.) collected in the New England area. The isoagglutinin titer of Fraction III suggested that these protein components were precipitated completely in Fraction III. As expected, ceruloplasmin and the larger part of the cholesterol content were also rendered insoluble in this fraction. Recent investigations on the distribution of cholesterol between α_1- and β_1-lipoproteins in pathological, traumatic and postmortem synovial fluid, involving the separation of the two groups of lipoproteins by flotation technique (20) and by paper electrophoresis (21), suggested a ratio of the α_1- to the β_1-lipoproteins of approximately 1:3, which is similar to that found in blood plasma. Thus, the cholesterol distribution reported in Table III is affected by the fact that some of the α_1-lipoproteins, usually rendered insoluble in Fraction IV, had probably been denatured and were, at least partly, precipitated earlier in Fraction III. Immunochemical analysis of Fraction III using the Ouchterlony technique revealed precipitin zones very similar to

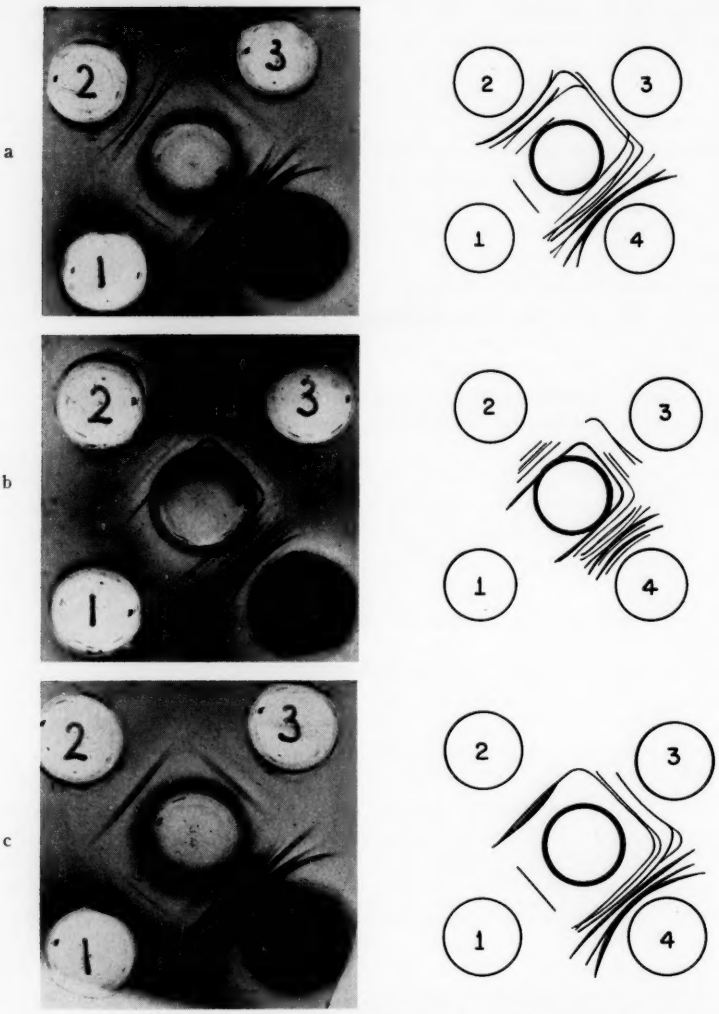

FIG. 2. OUCHTERLONY PLATES OF THE PROTEIN FRACTIONS FROM POSTMORTEM SYNOVIAL FLUID

In all plates Reservoir 1 contained the synovial fluid protein fraction to be analysed. Reservoir 2 was filled with the corresponding fraction derived from normal human plasma. Reservoir 3 contained β_1-metal combining protein. Reservoir 4 normal human plasma and the center reservoir horse serum against human serum. For the sake of clarity a sketch of each figure is given on the right hand side.

Fig. 2a—Fraction IV. After a diffusion time of two days a picture similar to Figure 2b was observed and is, therefore, not included here. After a diffusion time of nine days, Figure 2a was obtained.

Fig. 2b—Fraction V. Picture taken after a diffusion time of two days. The heavy line close to the center is due to serum albumin.

Fig. 2c—Fraction V. Picture taken after a diffusion time of nine days. The precipitin zone due to albumin in the center reservoir and no longer visible. Additional precipitin lines partly seen in Figure 2a formed now closed systems.

Fraction III was analyzed by the same method. After nine days six closed systems of precipitin lines were noted. The picture appeared similar to that of Fraction III of arthritic synovial fluid which has been reproduced in the previous publication (1).

those of the corresponding fraction of certain pathological joint fluids (1) and suggested the identity of the synovial fluid proteins with those of normal human plasma. *Fraction IV* was analyzed by the same technique. The formation of closed systems of precipitin lines (Figure 2a) again strongly suggested the identity of these proteins with those of normal human plasma. The capacity of the albumin present to bind 2-(4′ hydroxybenzeneazo-) benzoic acid (HBABA) (22) supported the latter finding (Table III). On the basis of the results obtained by fractionating postmortem and traumatic synovial fluid and normal and pathological human plasma, no indication could be obtained as to whether a particular α_2-globulin present in Fraction III or IV of plasma

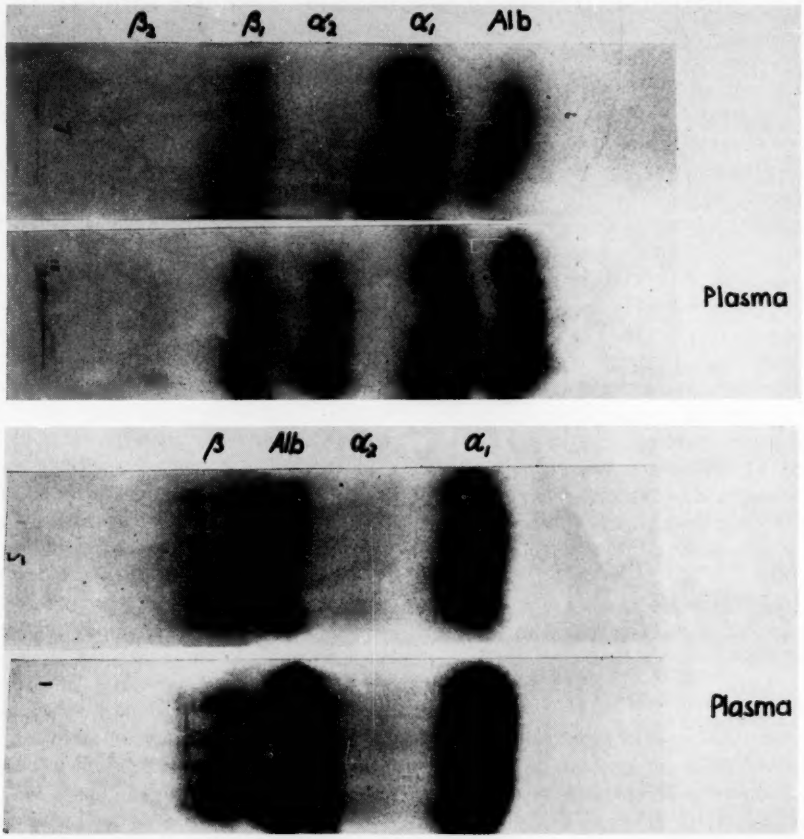

FIG. 3. PAPER ELECTROPHORESIS OF FRACTION VI DERIVED FROM POSTMORTEM HUMAN SYNOVIAL FLUID

Dark lines indicate the position of application. Electrophoretic movement of the proteins was from left to right towards the anode. Staining was done with Amidoschwarz 10B. For comparison Fraction VI of normal human plasma designated as "Plasma" was analyzed simultaneously. Top pictures represent the analysis at pH 8.6 in diethyl-barbiturate buffer, Γ/2 0.1. Bottom pictures represent the analysis in pH 4.5 acetate buffer, Γ/2 0.1. Due to the flow of buffer in the direction of the electric movement the albumin which is essentially immobile under the latter conditions was displaced to the right. Fraction VI contains albumin, low molecular weight acidic α_1- and α_2-glycoproteins and β_1- and β_2-globulins (11–13). The designations on the top and bottom pictures, Alb, α_1, α_2, β_1, and β_2, refer to the same proteins.

may have preferentially been excluded during the passage of the blood proteins through the synovial tissue. *Fraction V* was of yellow color, in contrast to the colorless quality of the corresponding plasma fraction, and consisted for the most part of albumin as judged by its solubility, sedimentation constant, electrophoretic mobility and relatively low content of protein-bound hexose. The relative capacity of the albumin present in this fraction to bind HBABA was 0.7. The corresponding value of Fraction IV was 1.0 and that of the pooled digested synovial fluid 1.3 as compared with a value of 1.0 for pure human serum albumin. The found deviations in the albumin determination obtained by this dye-binding method have already been reported (1) and were not significant as could be shown with known artificial protein mixtures. Immunochemical analysis of this fraction by the Ouchterlony method (Figures 2b and 2c) strongly supported the finding of the identity of the synovial fluid proteins with those of normal plasma. *Fraction VI* was composed of 110 mg. and was approximately fivefold the weight of the corresponding fraction derived from the same amount of normal plasma proteins. In addition paper electrophoresis at pH 8.6 and 4.5 (Figure 3) indicated a relatively increased amount of orosomucoid (11) and certain β_1-globulins and a relatively low content of the low molecular weight α_2-glycoproteins (13). Again a yellow color was noted in this fraction—thus, by the method of fractionation the proteins of postmortem joint fluid proved to be identical with those of blood plasma.

DISCUSSION

The characterization of the proteins of postmortem synovial fluid was carried out essentially in the same manner as presented in an earlier paper on the characterization of the proteins of pathological joint fluid (1). It was based to a large extent on methods which measure general and specific properties of blood plasma proteins. From the results obtained during this study it was concluded that essentially the total amount of the synovial fluid proteins is derived from blood plasma as judged by the employed criteria.

In the present investigation certain properties of postmortem synovial fluid reported by others were confirmed. The volume of fluid which could be aspirated from the knee joints of patients free from joint disease and edema, was found to vary within the range reported earlier by Ropes, Rossmeisl, and Bauer (3) and Coggeshall, Warren, and Bauer (4). The protein concentration of the fluid samples listed in Group 6 of Table II agrees with the corresponding value reported by Ropes and co-workers for postmortem (3) and Platt, Pigman, Holley, and Patton for normal human synovial fluid (8). For comparison it is of great interest to observe that the protein content of the interstitial fluid of muscle (23) and tissue fluid (24) is approximately the same. Most synovial fluid samples investigated during this study showed that the relative proportion of albumin was increased over that of certain pathological joint fluids (1, 10) and that of normal human plasma. A similar increase has previously been noted in traumatic synovial fluid (1, 10). However, the fluid volume found in traumatic arthritis is severalfold larger and the viscosity considerably lower than the corresponding values of postmortem joint fluid from patients free of joint disease. Further, the significantly elevated albumin-globulin ratio, particularly of the specimens of Group 6 in Table II, agrees with that of normal human synovial fluid (8), although it is difficult to evaluate exactly the significance of the percentage of albumin since some of our cases included patients with diseases often characterized by a lowering of the percentage of albumin in the blood. However, it is known that albumin which has a molecular weight lower than that of the average globulins, but approximately the same shape, permeates into the tissue about one and one-half times faster than the latter (24). The much more pronounced relative increase of α_1-acid glycoprotein (orosomucoid) concentrated in Fraction VI and characterized by an even lower molecular weight (45,000) but the same shape, is expected on the basis of these considerations. Furthermore, it is interesting to note that such fluids showed the calculated content of γ-globulin of 9 per cent (2).

The demonstration of hitherto unknown properties of postmortem synovial fluid, the consistent presence of the α_1- and α_2-globulins, the relative concentration of the α_2-globulins being consistently lower than that of the α_1-globulins, and of the α_1- and β_1-lipoproteins and of the severalfold increased amount of Fraction VI, are significant for

the characterization of joint fluid. Postmortem joint fluid is further characterized by the small quantity which can be aspirated from one knee joint (average, 1.0 ml), its gel-like appearance, the high protein concentration (3 per cent), and the low cholesterol-protein ratio (6 to 16 mg. per Gm.) and by the normal content of γ-globulin (9 per cent).

The results of our study further support the general finding that considerable amounts of plasma proteins of the body are in the extravascular system. Gitlin, Landing, and Wipple (25) recently demonstrated the presence of plasma proteins in various organs and tissues and, lately, Humphrey, Neuberger, and Perkins (26) reported on the presence of these proteins in skin and tendon.

SUMMARY

The proteins of postmortem synovial fluid from patients clinically free of signs or symptoms of joint disease have been characterized. As far as investigated they were found to be identical with those of blood plasma. The total amount of synovial fluid protein was essentially accounted for. This conclusion was based on the results obtained by the following methods: electrophoresis, immunoelectrophoresis, low temperature-low salt-ethanol fractionation and investigation of the obtained protein fractions by ultracentrifugation, electrophoresis, immunochemical analysis and for ion-binding capacity and other biological activities. Components produced solely by the synovial tissue are not excluded.

Taking normal human plasma as baseline, postmortem synovial fluid was further characterized by the following differences: relatively low protein concentration, decreased but distinct content of α_1- and β_2-lipoproteins, relatively increased content of albumin and orosomucoid, consistent presence of the α_1- and α_2-globulins, and the concentration of α_2-globulins being consistently lower than that of the α_1-globulins. Moreover, postmortem joint fluid contains a prealbumin component.

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ADDENDUM

The low molecular weight components noted in Fraction VI of postmortem synovial fluid have been further studied. On ultracentrifugal analysis a single peak with a sedimentation constant of approximately 1S was observed. As judged by the estimated, relatively high diffusion constant, the molecular weights of these constituents are probably below 15,000. Electrophoretic analysis (Tiselius apparatus) in pH 8.6, $\Gamma/2$ 0.1 barbiturate buffer of Fraction VI derived from hyaluronidase digested synovial fluid of young steers revealed four prealbumin components. Their respective electrophoretic mobilities were 13, 10, 9 and 8×10^{-5} cm.^{2}/Volt·sec. These substances were resistant to hyaluronidase, were not metachromatic, and did not bind protein stains. It is important to emphasize that these compounds did not occur in pathological synovial fluid.

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THE METABOLISM OF D–RIBOSE IN MAN

By STANTON SEGAL AND JOSEPH FOLEY

(From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, Department of Health, Education and Welfare, Bethesda, Md.)

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Previously thought to be primarily a structural component of nucleic acids, the pentose, D-ribose, in the form of the ester, ribose 5-phosphate, has now been shown to be an important intermediate in the pentose phosphate pathway of glucose metabolism. The sequence of the reactions of this pathway has been summarized by Horecker and Mehler (1) and Wood (2), and surveys of its significance in various mammalian tissues have been reported (3–6).

Naito (7) has investigated the glycogenic effect of ribose after its injection into mice and rabbits, and Herrmann and Hickman (8) have described the utilization of ribose by beef cornea. Horecker, Gibbs, Klenow, and Smyrniatis (9) have demonstrated the conversion of ribose to hexose with rat liver homogenates and Katz, Abraham, Hill, and Chaikoff (10), incubating ribose 1-C^{14} with rat liver slices, observed the formation of glucose with a pattern of labeling that was predictable from the reactions of the pentose phosphate pathway. Similar findings have been reported by Hiatt (11), studying the *in vivo* conversion of labeled ribose to glucose in the mouse. Recently Agranoff and Brady (12) have isolated and purified from calf liver an enzyme, ribokinase, which is capable of phosphorylating ribose to ribose 5-phosphate, thus permitting the sugar to enter the pentose phosphate pathway. The entrance of ribose into the pathway, and the sequence of its conversion to glucose are shown in Figure 1.

Since ribose appears to be of importance in mammalian metabolism, we have undertaken a study of certain phases of ribose metabolism in man. This paper presents data concerning the fate of ribose after its intravenous administration in both unlabeled and C^{14} labeled form to both normal and diabetic subjects. Preliminary results of these studies have been published (13), as well as a report of the hypoglycemia induced by ribose infusion (14). Previous publications (15, 16) from this laboratory have described the physiologic disposition in man of the related pentoses D-xylose, D- and L-arabinose, and D-lyxose, as well as the effects of insulin on the blood levels of these sugars.

MATERIALS AND METHODS

Part of the D-ribose used in these experiments was a generous gift of Dr. Hewitt G. Fletcher, Jr., of the Section on Carbohydrates, Laboratory of Chemistry of this Institute, and part was purchased from the Pfanstiehl Co., Waukegan, Ill. The sugar was pure, by the criteria of both optical rotation and paper chromatography. Seven and a half per cent sterile pyrogen-free solution of the sugar was used for infusion. Ribose 1-C^{14}, specific activity 2.01 μc. per mg. and 0.89 μc. per mg., was obtained from the National Bureau of Standards and was found to be solely ribose by chromatography and radioautography. Purified phosphoglucomutase prepared according to Najjar (17) was a gift of Dr. K. Kurahashi. The glucose 1-phosphate was commercial material electrophoretically freed of glucose 1, 6-diphosphate. Ribose 5-phosphate was obtained from Schwarz Laboratories and used as the sodium salt.

Twenty-seven intravenous infusions of various amounts of D-ribose were given to six fasting normal male and one female volunteers, 19 and 24 years of age, and to three subjects with diabetes mellitus. All of the normal subjects were maintained on a 250 Gm. carbohydrate diet prior to study. Diabetics received their last dose of crystalline insulin 24 hours before each experiment. Administration of the ribose solution was carried out either over a 15 minute period or, after a small priming dose, by constant infusions, using a Bowman pump. By the technique already described (15), blood specimens were obtained, those for glucose and ribose determination at 3 to 10 minute intervals, and those for phosphate and pyruvate analysis at 20 minute intervals. In eleven experiments 0.1 U per Kg. body weight of crystalline insulin was given intravenously in order to ascertain the effects of insulin on blood levels and urinary excretion of the pentose. Fractional urines were obtained for 24 hours and preserved by freezing.

Analytical procedures. Blood and urine glucose were estimated by employing the glucose oxidase method devised in this laboratory (15). Ribose was analyzed by the orcinol method (18), using blood filtrates and urine in which glucose had been destroyed by glucose oxidase. Serum inorganic phosphate was determined by the method

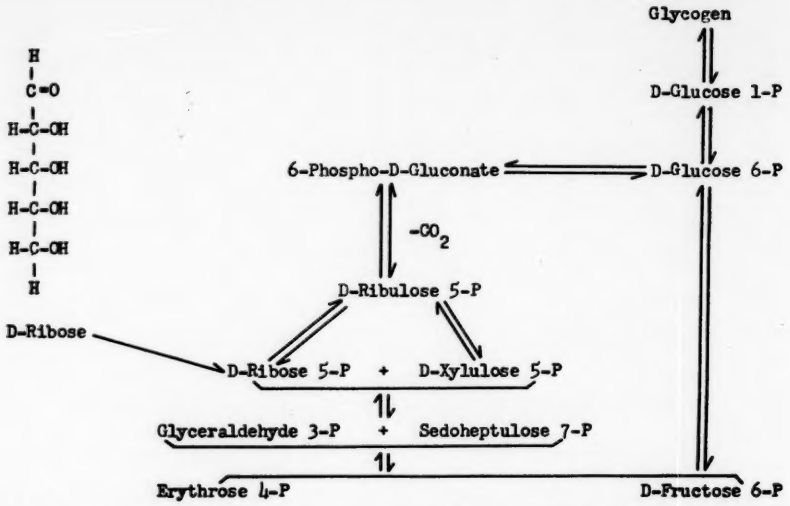

FIG. 1. THE SEQUENCE OF REACTIONS FOR THE ENTRANCE OF RIBOSE INTO THE PENTOSE PHOSPHATE PATHWAY AND ITS CONVERSION TO GLUCOSE

of Fiske and Subbarow (19) and blood pyruvate by the method of Segal, Blair, and Wyngaarden (20).

Expired CO_2 was collected and isolated as barium carbonate, as previously described (15). The CO_2 content of expired air was measured in a Cambridge CO_2 analyzer. Radioactivity in the CO_2 was determined by liberating the gas from barium carbonate and diffusing it by the method of Eisenberg (21) into hyamine base, which was counted by the technique of Passmann, Radin, and Cooper (22) in a liquid scintillation counter (Packard Co., La Grange, Ill.). The overall efficiency of CO_2 counting was 39 per cent. Cumulative excretion of $C^{14}O_2$ was estimated according to Berlin, Tolbert, and Lawrence (23).

Blood radioactivity was assayed in the following manner: Ten ml. of blood was added to an equal volume of 7 per cent perchloric acid for protein precipitation. An aliquot of resultant filtrate was neutralized with 5 N potassium hydroxide, and the precipitated $KClO_4$ centrifuged. A portion of the neutralized filtrate was then concentrated to 1.5 ml. under a nitrogen stream, and 0.5 ml. of this was added to 10 ml. of dioxane phosphor containing 5 per cent naphthalene and 0.8 per cent diphenyloxazole (24) for scintillation counting.

Urinary C^{14} counting was performed as in our previous communication (15).

Urine was chromatographed on Whatman No. 1 paper in a descending system for eight hours, employing methyl ethyl ketone, acetic acid, water, 6:1:1 as the solvent (R_F glucose, 0.07; ribose, 0.23). When tracer amounts of C^{14} pentose had been used, unlabeled ribose was added as a marker, the markers being visualized with analine hydrogen phthalate spray (25). Sugars were eluted by shaking paper strips with water in a small flask. The eluates were concentrated and assayed for activity in the same manner as whole urine. Recovery of sugars chromatographed in this was ranged from 93 to 103 per cent.

Glucose was isolated as crystalline potassium gluconate from the urine of the diabetic subject, who received C^{14} ribose by treating the urine according to the procedure of Moore and Link (26). The potassium gluconate was recrystallized twice from methyl alcohol. One portion of the gluconate was oxidized to CO_2 and counted as above, and another was degraded by the periodate method of Eisenberg (27), whereby carbon atoms one and six, individually, and two to five, collectively, could be isolated as CO_2 and counted.

The assay for phosphoglucomutase activity was that of Klenow (28).

RESULTS

The disappearance from blood of ribose administered intravenously

Semilogarithmic plots of decreasing blood ribose levels determined after the 15 minute infusion of various amounts of the sugar are shown in Figure 2. It is apparent that an entirely linear curve is obtained only when 3 Gm. amounts were injected, and that doses larger than 3 Gm. produced a lag phase before the onset of a linear disappearance. The deviation from a straight line of the blood ribose values in the lag phase does not represent a distribution gradient between blood and the total ribose compartment, for deviations due to this phenomenon lead to initial values higher than the linear curve. Likewise, the deviation is not due to a distribution gradient of ribose between

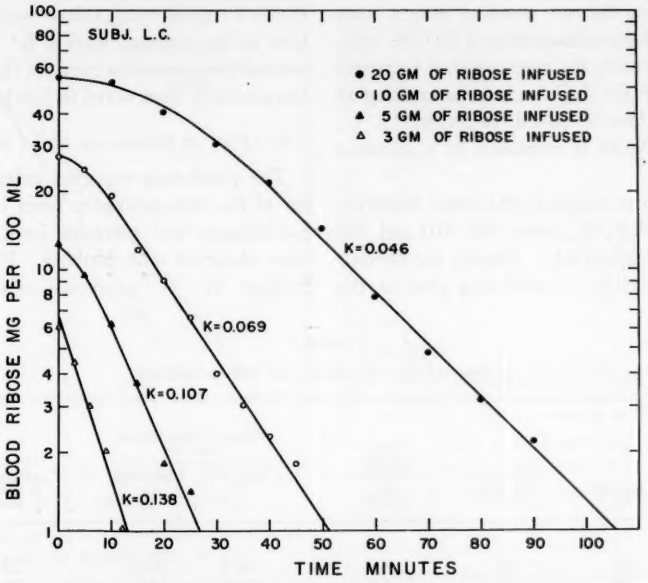

FIG. 2. SEMILOGARITHMIC DISAPPEARANCE CURVES OF RIBOSE FROM BLOOD AFTER 15 MINUTE INFUSION OF VARIOUS AMOUNTS OF PENTOSE (SUBJECT L. C.)

plasma and red blood cells, for experimentally determined ribose concentrations were essentially the same in whole blood and plasma during this early phase. These results indicate that the removal of ribose from blood is a first order kinetic process only when the ribose blood level is below some critical value.

Although developed for the analysis of substrate enzyme relationships, the mathematical treatment of Lineweaver and Burk (29) has been applied to the more complex problem of sugar transport across cell membranes (30). This type of analysis has been applied here to the clearance of ribose from blood. Using the data from the studies in Subject L. C. (Figure 2) and plotting the reciprocal of the initial disappearance rate in mg. per 100 ml. per minute versus the reciprocal of the blood ribose concentration in mg. per 100 ml., one obtains the straight line curve shown in Figure 3. From the curve, the K_s, or ribose concentration, at the point of half saturation of the clearance system, can be derived. The K_s is 10 mg. per 100 ml. Thus the system is completely saturated at initial blood levels of 20 mg. per 100 ml., and linear curves should be obtained when the blood level is below this value.

In Table I are given the proportional rate constants for the disappearance of ribose from blood, as derived from a knowledge of biological halftime, and expressed in terms of per cent of ribose disappearing per minute, K^1 equals K times 100. In

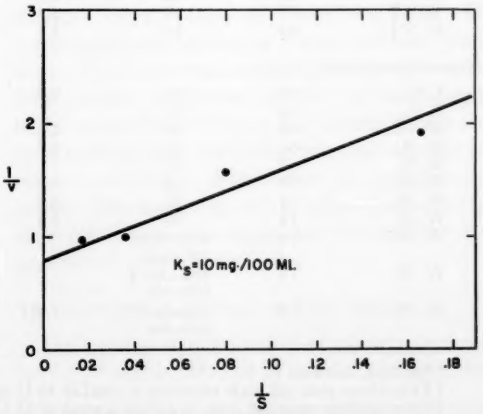

FIG. 3. GRAPH OF THE RECIPROCAL OF THE INITIAL VELOCITY IN MILLIGRAMS PER 100 MILLILITERS PER MINUTE VERSUS THE RECIPROCAL OF THE INITIAL CONCENTRATION IN MILLIGRAMS PER 100 MILLILITERS OBSERVED AFTER THE INFUSION OF VARIOUS DOSES OF RIBOSE IN SUBJECT L. C.

the normal subject, the rate constant ranges from 3.8 to 5.8 per cent per minute after a 20 Gm. dose. As the dose decreases, the rate constant increases to as high as 13.8 for 3 Gm. It is interesting to note that as the dose is halved, in Subject L. C., Figure 1, the K value is increased by a constant factor of 1.5.

The blood glucose values in the three diabetics, C. M., M. S., and N. N., were 470, 310 and 293 mg. per 100 ml., respectively. Despite the obvious lack of adequate ability to metabolize glucose, the ribose disappearance curve was identical in pattern to the normal, with a K^1 of 4.6 to 5.1. A normal disappearance curve of the pentose D-xylose has similarly been noted in Subject C. M. (31).

The effect of insulin on blood ribose levels

The previously reported enhancement by insulin of the disappearance from blood of D-xylose, L-arabinose and D-lyxose from blood (16) has been observed with D-ribose. Figure 4 reveals in Subject W. W. a control ribose disappearance

TABLE I

Ribose infusion in normal and diabetic subjects

Subject	Weight *Kg.*	Quantity infused *Gm.*	Disappearance from blood K^1 *%/min.*	Volume of distribution based on: Observed $C_0{}^1$* *L.*	Volume of distribution based on: Calculated $C_0{}^1$ *L.*	Urinary recovery *% admin. dose*	Time of insulin admin. relative to end of infusion *minutes*
Normals							
W. Y.	81	20	3.8	24.9	19.0	22	
W. W.	74	20	4.8	21.5	10.7	20	
W. W.	74	20	4.8	19.3	10.7	21	
J. D.	81	20	5.0	26.1	19.0	32	
J. D.	81	20	5.8	21.5	3.8	21	
L. C.	80	20	4.6	29.1	11.0	26	
L. C.	80	10	6.9	29.6	21.7	18	
W. B.	64	10	8.2	21.5	7.9		
J. D.	81	10	10.5	19.6	5.5	17	
L. C.	80	5	10.7	31.7	22.2	14	
L. C.	80	3	13.8	40.0	36.3		
J. D.	81	3	11.9	24.3	24.8		
Diabetes mellitus							
C. M.†	60	20	4.6	22.3	9.2	24	
M. S.‡	73	20	5.1	24.4	10.5	30	
N. N.§	60	20	5.1	20.8	6.7	24	
Normal plus insulin							
L. C.	80	10	9.3			17	−10
C. C.	66	10				14	+25
W. R.	66	20	4.3			18	+30
W. R.	66	20	4.5			18	+30
W. B.	64	20				23	+20
W. W.	74	20	6.0			16	+20
W. W.	74	20	6.0			18	− 7
W. W.	74	20	5.8			21	+ 2
W. W.	74	constant‖ infusion				12	+60††
W. W.	74	constant¶ infusion				8	+70††
W. W.	74	constant** infusion	7.0‡‡			5	+70††

* See text, footnote 1.
† Fifty-three year old male requiring a total of 36 U of globin and crystalline insulin daily.
‡ Twenty-three year old male requiring a total of 45 U NPH and crystalline insulin daily.
§ Thirty-one year old male requiring a total of 45 U of NPH and crystalline insulin daily.
‖ Constant infusion 137 mg. per minute after a 4 Gm. prime (Figure 5).
¶ Constant infusion 146 per minute after a 4 Gm. prime.
** Constant infusion 151 mg. per minute after a 4 Gm. prime.
†† Insulin injected at stated time after the start of the constant infusion.
‡‡ Calculated from data obtained for decreasing blood levels after cessation of the constant infusion.

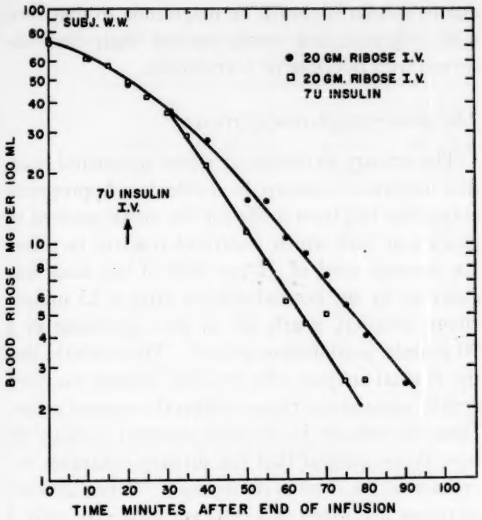

FIG. 4. THE EFFECT OF INTRAVENOUS INSULIN ON THE DISAPPEARANCE OF RIBOSE FROM BLOOD

curve, as well as one after insulin administration. As a result of the insulin injection, an earlier onset of the linear phase and an increase in the rate constant of disappearance were seen. In another experiment in Subject W. W. (Table I), insulin was injected during the ribose infusion so that its effect might be observed at high blood ribose values.

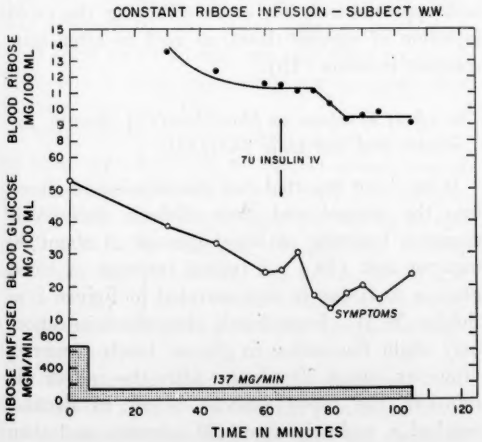

FIG. 5. BLOOD LEVELS OF RIBOSE AND GLUCOSE OBSERVED DURING THE CONSTANT INFUSION OF RIBOSE
Effects of intravenous insulin administration.

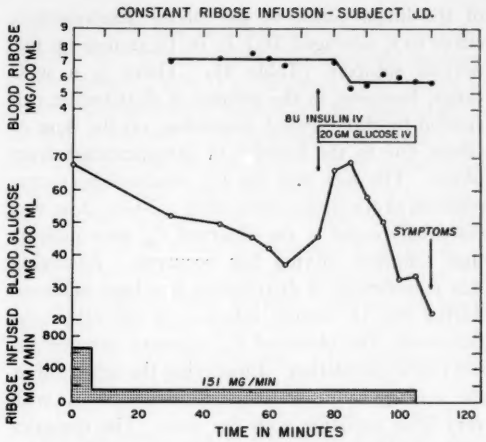

FIG. 6. BLOOD LEVELS OF RIBOSE AND GLUCOSE OBSERVED DURING THE CONSTANT INFUSION OF RIBOSE
Effects of intravenous insulin and intravenous glucose administration.

No clear effect was seen, however, until the level had fallen to about 30 mg. per cent, at which point the curve deviated from the control and became superimposable on the insulin curve shown.

Because of the unusual kinetics of ribose disappearance and the rapid decline in blood levels, the effect of insulin was tested on ribose blood levels maintained at a low constant value by continuous ribose infusion, a technique used previously (16). Figures 5 and 6 demonstrate these studies. After insulin, the ribose level falls, with a subsequent attainment of a new constant blood level. The intravenous administration of a sufficient quantity of glucose to raise the blood sugar to the control level (Figure 6) did not prevent the insulin effect, thus indicating that the response of ribose to insulin is independent of hypoglycemia *per se*. The symptoms of hypoglycemia occurring after insulin injection were not alleviated by the presence of ribose in the body.

The distribution of ribose in body fluids

The volume of distribution of ribose calculated from C_0' [1] values obtained by graphic extrapolation

[1] The concentration of ribose in plasma water (C') has been calculated from the analytical value on whole blood (C) divided by 0.8, the approximate water content of blood. Volume of distribution equals Q/C_o' in which C_o' equals $C_o/0.8$, the concentration of ribose in

of the linear phase of the ribose disappearance curve to t_0 averaged 16.1 L. in 12 studies on five normal subjects (Table I). There is a wide range, however, in the volume of distribution calculated by this method, depending on the dose of ribose due to the kinetics of disappearance from blood. The fact that the C_0' obtained by extrapolation of the decay curve after a 3 Gm. dose was essentially equal to the observed C_0' may indicate that complete mixing has occurred. Assuming that equilibrium of distribution had been achieved during the 15 minute infusion in the other experiments, the observed C_0' appears suitable for use in the calculation. Employing the latter value, the average value of distribution is 25.8 L. with very little variation with the dose. The diabetics appear to have a normal ribose "space," as determined by both of these methods of estimation.

Calculations of the above type present the inherent difficulty of estimating the amount present in the body at time zero, the theoretical moment of mixing, as well as the concentration at that time, mainly due to the fact that ribose has a metabolic fate other than simple distribution and excretion. Under these circumstances, a preferable type of calculation is that employed by us for determining the volume of distribution of xylose (16) and based on the theoretical development of Dominguez, Goldblatt, and Pomerene (32, 33) and others (34). This treatment requires the constant infusion type of experiment shown in Figures 5 and 6. From knowledge of the concentration of ribose in blood at equilibrium, the composite rate of loss of ribose from the compartment, which is equal to the rate of infusion, and the rate constant of ribose disappearance from blood as determined following the completion of the infusion, the volume of distribution for Subject W. W. was calculated to be 14 L. before insulin administration. Since this individual weighed 74 Kg., the ribose compartment was equal to 19 per cent of body weight, a distribution essentially in extracellular fluid. This finding parellels that for D-xylose (15).

The volume of the ribose compartment after insulin was calculated to be 16.6 L. or 22.4 per cent of body weight. This increase in ribose "space" due to insulin is similar in magnitude to that seen with D-lyxose, but much smaller than that observed with D-xylose or L-arabinose.

plasma water at the theoretical moment of mixing, and Q is the quantity infused (15).

The urinary excretion of ribose

The urinary excretion of ribose as orcinol reactive material is shown in Table I. Appropriate correction has been made for the small amount of glucuronic acid which is orcinol reactive in urine. An average total of 21 per cent of the dose was excreted by the normal subject after a 15 minute ribose infusion, nearly all of this appearing in a 90 minute postinfusion period. The diabetic and the normal subject who received insulin excreted renally amounts of ribose within the control range. Data on Subject L. C. who received various ribose doses suggest that the urinary excretion decreases as the dose is diminished. When 20 Gm. of ribose was given at a constant slow rate, only 5 to 12 per cent was found in the urine. This appears to be a consequence of the slow infusion rate, and is not related to the insulin given during these experiments. In one such experiment, urine collected prior to insulin injection revealed a urinary loss of 8 mg. per minute, while 146 mg. per minute was being infused. This excretion rate of 5.5 per cent per minute approximates very well the total excretion of 5 per cent of the dose. These results are in contrast to those obtained with D-xylose. The total urinary loss of this pentose was approximately the same following the rapid injection of various doses, as well as after slow constant infusion (16).

The effect of ribose on blood levels of glucose, pyruvate and inorganic phosphate

It has been reported that the infusion of ribose into the normal and some diabetic individuals causes a lowering of blood glucose of about 30 mg. per cent (14). A typical response of blood glucose to ribose is demonstrated in Figure 7 in Subject W.W. Immediately after ribose injection, only slight fluctuation in glucose levels occurred. However, about 30 minutes after the end of the infusion, the blood glucose began to decline, reached a nadir in about 30 minutes, and then returned to approximately the control level. The effect was observed when blood ribose values had decreased to low levels. This hypoglycemic effect

TABLE II

Change in blood glucose and phosphate after infusion of various doses of ribose

Subject	Amount infused *Gm.*	Control blood glucose *mg. per 100 ml.*	Minimal blood glucose *mg. per 100 ml.*	Decrease in inorganic phosphate *mg. per 100 ml.*
L. C.	20	71	30	
L. C.	10	84	50	0.6
L. C.	5	67	61	0.9
L. C.	0 (saline)*			
J. D.	20	63	37	0.3
J. D.	20	91	59	
J. D.	10	53	45	1.2
J. D.	13.7†	68	37	
J. D.	0 (saline)*			0.3

* Two hundred sixty-seven ml. of normal saline was infused for 15 minutes and blood for phosphate determination was obtained at 20 minute intervals.

† Four Gm. prime followed by constant infusion of 151 mg. per minute.

of ribose is shown again in the same subject (Figure 5) after a 4 Gm. priming dose and the constant infusion of ribose at a slow rate.

Table II reveals the effect of different doses of ribose on blood glucose values in two subjects. A marked reduction in blood glucose occurred after 20 and 10 Gm. doses, with but a questionable change after 5 Gm. in Subject L. C. Subject J. D.'s blood glucose fell reproducibly after 20 Gm. infusions but decreased only slightly after 10 Gm. was given. In contrast to the latter result, when Subject J. D. was given ribose by constant infusion (Figure 6), his blood glucose levels had fallen from 68 to 52 mg. per cent after receiving 8 Gm. and to a low value of 37 mg. per cent after receiving only 13.7 Gm.

Though blood glucose values as low as 15 mg. per cent were observed, no subject complained of hypoglycemic symptoms, and only in Subject W. W. could some slight signs (blanching of lips and a peculiarity of gaze) be detected. When 0.1 U per Kg. of insulin was given during the ribose-induced hypoglycemia (Figure 5), greater hypoglycemia occurred, and frank symptoms were then observed.

A fall in serum inorganic phosphate was consistently seen after ribose infusion (seven studies). In four normals receiving 20 Gm. the decrease ranged from 0.3 to 0.7 mg. per cent. One diabetic showed a decline of 0.6 mg. per cent. The significance of these results appears to be open to question when one observes the blood phosphate changes in a given subject who received various ribose doses (Table II). Subject L. C. shows a greater phosphate fall after a 5 Gm. than after a 10 Gm. dose, and J. D., a greater fall after a 10 Gm. than after a 20 Gm. dose. Both individuals respond to saline administration with a 0.3 mg. per cent fall in phosphate, this being equal to the response of J. D. to a 20 Gm. infusion of ribose.

The blood pyruvate changes observed after 20 Gm. infusions in four normals and one diabetic were variable and insignificant, ranging from -0.28 to $+0.10$ mg. per cent. The relationship of inorganic phosphate and pyruvate to glucose and ribose levels is shown in Figure 7. Worthy of note is a comparison of the effects of ribose and insulin on the blood phosphate and pyruvate in a given individual. In the experiment shown in Figure 5, the phosphate had fallen 0.5 mg. per cent and the pyruvate was unchanged from the control value at the time when the blood glucose had decreased to 23 mg. per cent (60 minutes). The

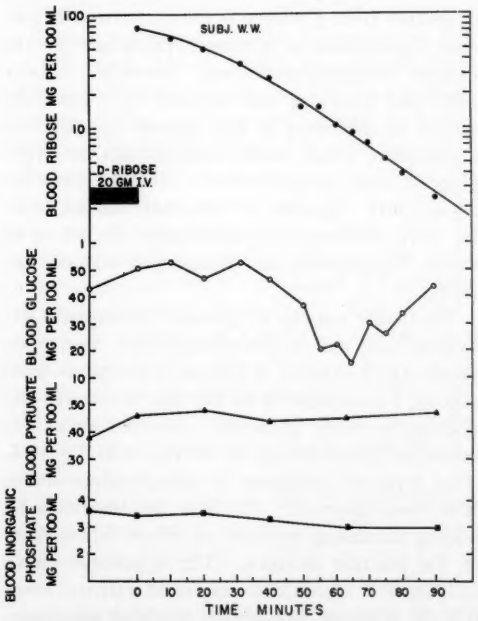

FIG. 7. THE EFFECT OF THE ADMINISTRATION OF RIBOSE ON BLOOD GLUCOSE, PYRUVATE AND SERUM INORGANIC PHOSPHATE LEVELS

A significant decrease in blood glucose level is noted.

injection of insulin and concomitant fall in glucose was followed by a further fall in phosphate of 1 mg. per cent and a rise of blood pyruvate from 0.51 mg. per cent to 1.40 mg. per cent within 28 minutes. The lowest blood glucose value after insulin in this study is about equal to the lowest value seen after ribose in the same subject (Figure 7) where little change occurred in phosphate or pyruvate values.

Studies with phosphoglucomutase

The lack of a significant increase in blood pyruvate and questionable decrease in serum inorganic phosphate, accepted parameters for indicating increased peripheral glucose utilization (35–37), suggested that the ribose-induced hypoglycemia was not due to an insulin-like effect. Quantitative determination of urinary glucose after ribose infusion (14) eliminated glucosuria as a cause and indicated that the hypoglycemia might be the result of a defect in the hepatic homeostatic mechanism for maintenance of blood glucose.

One of the important reactions in the formation of glucose from glycogen is the conversion of glucose 1-phosphate to glucose 6-phosphate by the enzyme phosphoglucomutase. Recently, Sidbury (38) and Ginsburg and Neufeld (39) have observed an inhibition of this enzyme by galactose 1-phosphate, a fact which could explain the hypoglycemia seen in galactosemics after galactose infusion (40). Because of these observations, studies were performed to demonstrate the effect of ribose 5-phosphate on phosphoglucomutase activity.

The assay system of glucose 6-phosphate dehydrogenase and triphosophopyridine nucleotide detects the formation of glucose 6-phosphate from glucose 1-phosphate by an increase in reduced triphosophopyridine nucleotide recorded as an increase in optical density at 340 mμ as in Figure 8. Two types of inhibition of phosphoglucomutase have been observed. The first can be shown by adding increasing amounts of ribose 5-phosphate to the reaction mixture. The uppermost curve in Figure 8 shows the enzymatic activity when 0.5 μM of ribose 5-phosphate is added simultaneously with the substate glucose 1-phosphate. This curve represents a 5 per cent inhibition of the enzyme. When 2.5 μM and 5 μM of ribose ester are added, one obtains a 59 and 72 per cent inhibition, respectively. We believe this to be an ion effect of ribose 5-phosphate, for Cori, Colowick, and Cori (41) and Klenow (42) have shown the enzyme to be sensitive to changes in salt concentration of both inorganic ions and organic phosphates.

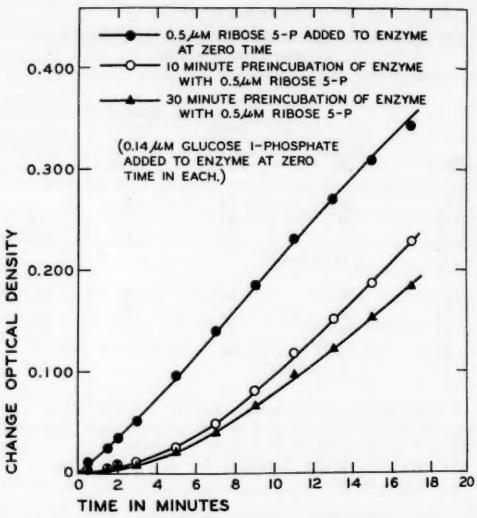
0.5 μM RIBOSE 5-P ADDED TO ENZYME AT ZERO TIME
10 MINUTE PREINCUBATION OF ENZYME WITH 0.5 μM RIBOSE 5-P
30 MINUTE PREINCUBATION OF ENZYME WITH 0.5 μM RIBOSE 5-P
(0.14 μM GLUCOSE 1-PHOSPHATE ADDED TO ENZYME AT ZERO TIME IN EACH.)
CHANGE OPTICAL DENSITY
0.400
0.300
0.200
0.100
0
0 2 4 6 8 10 12 14 16 18 20
TIME IN MINUTES

FIG. 8. THE INHIBITION OF PHOSPHOGLUCOMUTASE BY RIBOSE 5-PHOSPHATE

The ordinate represents the optical density change at 340 mμ due to the formation of reduced triphosphopyridine nucleotide by the action of glucose 6-phosphate dehydrogenase on the glucose 6-phosphate formed by the mutase reaction.

The second type of inhibition can be shown by preincubation of the enzyme with the ribose ester. The results of this preincubation experiment are shown in Figure 8. Here is seen a profound initial inhibition of the mutase reaction which is eventually overcome. Klenow and Emberland (43) have demonstrated that phosphoglucomutase will convert ribose 5-phosphate to ribose diphosphate. Since, to be active, the enzyme must be in a phosphorylated state, the inhibition seen in this type of experiment may be interpreted as a dephosphorylation of the enzyme during the preincubation period due to ribose diphosphate formation.

The fate of C^{14} after injection of ribose-1-C^{14}

In an effort to obtain information concerning the metabolic disposition of ribose, two studies

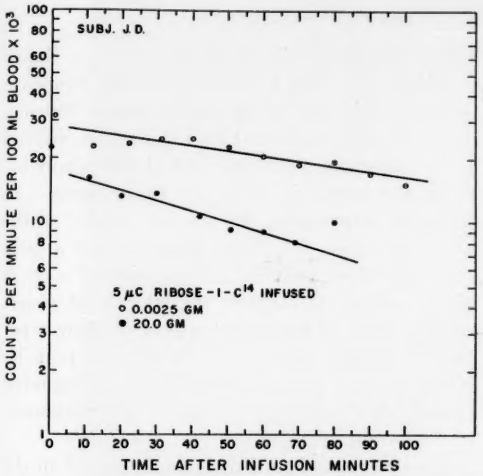

FIG. 9. A SEMILOGARITHMIC PLOT OF THE C^{14} DISAPPEARANCE FROM BLOOD AFTER RIBOSE-1-C^{14} INFUSION BOTH AS TRACER AND LOAD DOSES

were carried out in one normal individual in which the fate of the label was determined following intravenous administration of ribose-1-C^{14}. In one experiment, 5 μc. of ribose-1-C^{14} was infused as a tracer dose (2.5 mg.). In the other, 5 μc. of the labeled material plus 20 Gm. of unlabeled ribose were infused from the same flask. The disappearance of C^{14} from blood, and the appearance of the label as expired $C^{14}O_2$, as well as the excretion of the label in urine were determined.

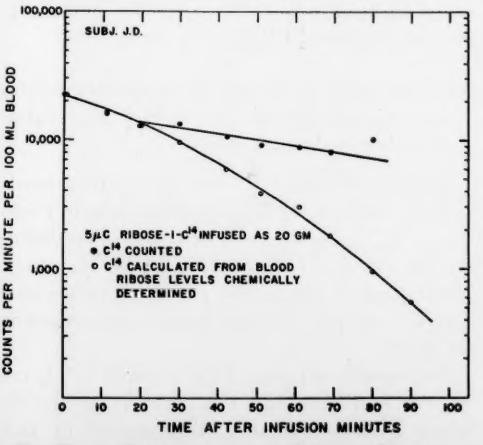
SUBJ. J.D.
COUNTS PER MINUTE PER 100 ML BLOOD
5μC RIBOSE-1-C¹⁴ INFUSED AS 20 GM
● C¹⁴ COUNTED
○ C¹⁴ CALCULATED FROM BLOOD RIBOSE LEVELS CHEMICALLY DETERMINED
TIME AFTER INFUSION MINUTES

FIG. 10. A COMPARISON OF THE OBSERVED DISAPPEARANCE OF C^{14} FROM BLOOD AND THAT CALCULATED FROM CHEMICALLY DETERMINED RIBOSE LEVELS

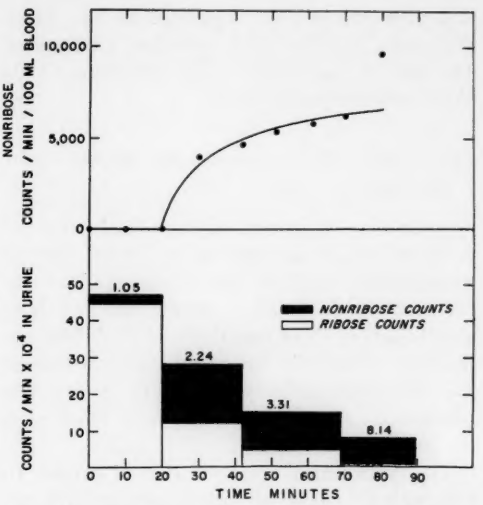

FIG. 11. THE SIMULTANEOUS APPEARANCE OF NONRIBOSE C^{14} IN BLOOD AND URINE

Numbers above bars indicate the ratio of total C^{14} counts to ribose counts in urine.

The disappearance of C^{14} from blood

Figure 9 demonstrates the disappearance curve of C^{14} from blood plotted semilogarithmically. The linear curves denote that C^{14} disappears according to first order kinetics. From the biologic halftime of disappearance, the rate constant of disappearance for the tracer dose and the 20 Gm. dose have been calculated to be 0.61 and 1.16 per cent per minute, respectively. These small rate constants are in marked contrast to the rather large ones observed for the linear disappearance of ribose chemically determined. Also worthy of note is the fact that the C^{14} decay constant for the larger dose is about three times as great as that for the tracer, an observation which differs from that noted for orcinol-determined ribose, namely that the rate constant is inversely proportional to the dose.

The constrast between the C^{14} and chemical disappearance curves for the load (20 Gm.) study is shown in Figure 10.[2] All of the data indicate

[2] The total C^{14} activity values of the curve based on chemically determined ribose were calculated by multiplying the analytical value in mg. per cent by the specific activity of ribose at zero time $\frac{(C^{14}\text{ counted})}{(\text{mg. per cent})}$ assuming the specific activity of ribose remained constant during the period of study.

that after ribose-1-C^{14} injection, as the ribose disappears from the blood, nonribose C^{14} appears. The rate of appearance of this nonribose C^{14} in blood is shown in Figure 11.

The urinary C^{14} excretion after labeled ribose injection

A total of 10 per cent of the C^{14} administered in tracer dosage appeared in the urine, whereas 40 per cent of the 5 μc. dose given as a load was excreted via this route. During the first hour after injection, about four times as much label appeared in urine in the latter study as in the former. This difference probably explains the greater rate constant for C^{14} disappearance from blood after the 20 Gm. dose.

The concomitant administration of carrier ribose with C^{14} ribose permitted comparison of urinary excretion of C^{14} and orcinol reactive material. Whereas the cumulative urinary excretion of C^{14} was 40 per cent, only 20 per cent of administered ribose as measured by orcinol was excreted by this route. The appearance of orcinol reactive material and total C^{14} in urine specimens obtained during the 90 minute postinfusion period is shown by the bar graph in Figure 11. The numbers above each bar represent the ratio of the total C^{14} activity to that due to orcinol-determined ribose. It is quite clear that with time, an increase in the proportion of total counts to ribose counts takes place, a phenomenon which parallels the increasing nonribose counts in blood.

Urine from these studies was chromatographed on paper, and the ribose area eluted and counted. Urine collected during the first hour after the tracer dose was chosen for study, since it contained nearly all of the C^{14} excreted. It was found that 92 per cent of the C^{14} eliminated after the tracer dose was unaltered ribose. A similar study of urine from the load experiment (20 to 42 minute period, Figure 11) revealed that only 42 per cent of the C^{14} applied to the paper could be located in the ribose band. This gives a ratio of total counts to ribose counts of 2.4 corroborating the ratio calculated (2.24) from urine counting and chemical data. The nature of the metabolite or metabolites in this urine remains to be identified.

Excretion of ribose as $C^{14}O_2$

The specific activity of expired $C^{14}O_2$ is shown in Figure 12. The CO_2 is appreciably labeled 5 minutes after the end of the 15 minute infusion period in both tracer and load studies on Subject J. D. However, there are marked differences between the curves in these two experiments. In the tracer experiment, the specific activity of the $C^{14}O_2$ increased at a faster rate, reached a peak about 40 minutes earlier, and maintained a much higher activity throughout the interval of observation. Both of the curves appear to show a period of constant specific activity after the peak labeling has been reached, a finding not encountered in studying the $C^{14}O_2$ excretion after the administration of other labeled pentoses (44).

The determination of the cumulative C^{14} in the expired air for six hours revealed 48 per cent of the tracer C^{14} but only 16 per cent of the C^{14} given in 20 Gm. Since a large quantity of C^{14} was found in the urine when the 20 Gm. was injected, however, comparison of the amount of metabolism to $C^{14}O_2$ may better be based not on the dose, but on the dose corrected for the six hour urinary excretion. When this is done, it is seen that 50 per cent of the retained activity is converted to CO_2 six hours after the tracer dose and 24 per cent after the larger dose. It seems apparent when comparing the $C^{14}O_2$ and urinary C^{14} excretion data above that when a load of ribose is given, a large quantity of metabolite is excreted in urine with reduction in the amount appearing as expired $C^{14}O_2$.

The metabolism of ribose-1-C^{14} in diabetes mellitus and evidence for the existence of the pentose phosphate pathway in man

Five μc. of ribose-1-C^{14} was given intravenously to a 53 year old, 60 Kg., unstable diabetic (Subject C. M., Table I) whose last dose of crystalline insulin was given 24 hours prior to the study. Blood glucose during the period of observation was 475 mg. per cent and acetone was present in the urine.

The specific activity of the expired $C^{14}O_2$ can be seen in Figure 12. Compared to results obtained in the tracer study in the normal (J. D.), the specific activity of the $C^{14}O_2$ is much lower, and the shape of the curve greatly altered. In the

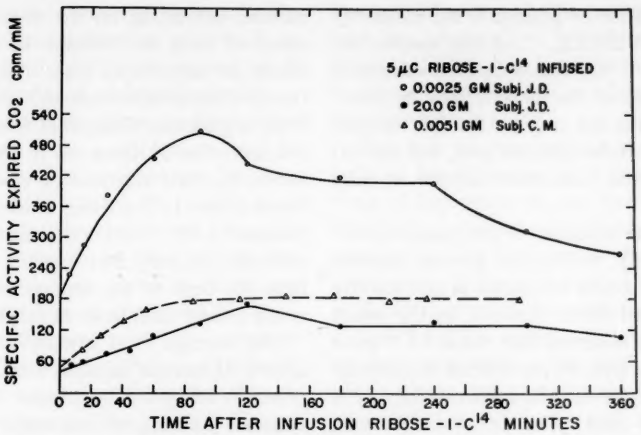

FIG. 12. THE SPECIFIC ACTIVITY OF EXPIRED $C^{14}O_2$ AFTER THE INFUSION OF RIBOSE-1-C^{14}

diabetic, the labeling of CO_2 reaches a low maximum and then remains constant throughout the period of observation. In five hours, the cumulative C^{14} excretion was only 13 per cent of the retained activity (administered dose of C^{14} corrected for urinary excretion). The difference from the normal in the specific activity of $C^{14}O_2$ and the low cumulative excretion of C^{14} as CO_2 from ribose-1-C^{14} in the diabetic are not reflected in the disappearance curve of ribose from blood. The latter is unaltered from the normal in this subject and in the other diabetics tested (Table I), and suggests that the defect in the conversion of the C^{14} of ribose to $C^{14}O_2$ in the diabetic occurs at a stage later than the clearance of the sugar from blood.

Urine was collected for 24 hours, hourly specimens being obtained for the first 5 hours. Twenty-eight per cent of the C^{14} administered was excreted via this route in 5 hours, and 35 per cent in 24 hours. Ribose and glucose in the hourly urine specimens were separated by paper chromatography, eluted from the paper and then counted. All of the radioactivity applied to the paper was found to reside in the ribose and glucose areas after development of the chromatogram. The cumulative excretion of C^{14} in ribose and glucose in the diabetic urine is shown in Figure 13. Five hours after ribose-1-C^{14} injection, ribose C^{14} excretion in the urine had almost ceased, the urinary pentose accounting for only 10 per cent of the C^{14} dose. Eighteen per cent of the administered label resided in glucose at this time. The 10 per cent excretion of C^{14} as ribose is essentially the same as that observed in the normal. It thus appears that the large amount of glucosuria occurring in this subject did not cause an increase in ribose excretion.

The specific activity of the urinary glucose was calculated and found to be essentially constant at 25 counts per minute per mg. throughout the first five hours. Since the urinary glucose is derived from the body pool of glucose, one may assume that all of the glucose in the body has this specific activity. Assuming also a glucose space of 17 per cent of body weight (45), and knowing the glucose concentration in plasma water,[1] a glucose pool of approximately 59 Gm. can be calculated for this diabetic. If this glucose is labeled to the extent of 25 counts per minute per mg., then approximately 30 per cent of the ribose C^{14} injected (5 million counts) is present in the body as glucose at any time during the first five hours. Almost 70 per cent of the administered label has been accounted for (28 per cent in urine, 30 per cent in body glucose, and 10 per cent in expired CO_2) five hours after injection.

The fact that the specific activity of body glucose is essentially constant for five hours may explain the constant specific activity obtained for expired CO_2 (Figure 12). It would appear that ribose is converted rapidly to glucose

and that the oxidation of glucose is the means by which C^{14} appears in CO_2. The low specific activity of expired $C^{14}O_2$ and decreased excretion of C^{14} in CO_2 seen in the diabetic may be interpreted as being due not only to the low specific activity in an expanded glucose pool, but also to the defect in glucose metabolism known to exist in the diabetic.

According to the reactions of the pentose phosphate pathway (1), radioactive glucose derived from ribose-1-C^{14} should be labeled predominantly in carbons one and three. Indeed, in the intact mouse, Hiatt (11) observed that ribose-1-C^{14} gave rise to C^{14} glucose with 34 per cent of the activity in carbon one, 56 per cent in carbon three and a small quantity of activity in the other carbons. In order to determine whether ribose-1-C^{14} was converted to glucose by this pathway in man, glucose, from urine obtained during the fourth hour after ribose injection, was isolated as crystalline potassium gluconate. An aliquot was converted to CO_2 to obtain the total radioactivity in carbon one to carbon six collectively. Another portion was degraded so as to obtain carbon one and carbon six, individually, and carbon two to carbon five, collectively, as CO_2. Thirty-one per cent of the activity in carbon one to carbon six resided in carbon one, 6 per cent in carbon six and the remainder in the carbon two to carbon five fraction, presumably the activity being in carbon three. These figures correspond with Hiatt's results in the mouse, and give evidence that ribose was metabolized in man by conversion to glucose via the pentose phosphate pathway.

DISCUSSION

The kinetics of the disappearance of D-ribose from blood differ from those of D-glucose (46) or D-xylose (15) by being dose dependent. Our kinetic data indicate that the system for clearing ribose from blood is saturated after the infusion of small amounts of the sugar. That the assumption has some validity may be derived from the expired $C^{14}O_2$ data after tracer and 20 Gm. dose of labeled ribose. With the larger dose, the maximum specific activity of the $C^{14}O_2$ appeared at a later time and reached a much lower value (Figure 12).

The rate constants of disappearance of ribose from blood ranged from 3.8 to 13.8 per cent per minute, depending on the dose. Rate constants observed after the infusion of small amounts of ribose are larger than those observed after the infusion of various hexoses and other pentoses (15). Only D-galactose disappears from blood at a rate similar to that of ribose (47). At comparable dose levels, the rate constant of ribose removal from blood is four to five times as large as that of other pentoses (15). Helmreich and Cori (48) have similarly reported that injected ribose disappears from the body of the nephrectomized rat at rates much greater than those of other pentoses.

The average total urinary excretion of ribose after a 15 minute infusion was 21 per cent of the dose. A much smaller amount was excreted after constant infusion of the sugar at a slow rate. None of our studies was designed to determine accurately the renal mechanism for excreting ribose, and we are not aware of any careful studies of the renal handling of this pentose comparable to those reported for xylose (49). One difference between the renal handling of ribose and xylose is suggested from the present studies. Plasma glucose levels of about 300 mg. per cent cause a saturation of the xylose tubular reabsorbing mechanism and increase the renal clearance of xylose (50). No such effect was observed in the ribose studies in diabetics, for with blood glucose levels of about 300 mg. per cent, these subjects excreted no greater amounts of ribose in urine than the subjects with normal blood glucose levels.

Normally, small amounts of ribose can be detected in urine (51). There have been several reports of a ribosuria associated with muscular dystrophy (52) and neoplastic disease (53). Whether this ribosuria is a primary defect in these diseases, or is merely a consequence of tissue breakdown has not been established definitely.

The present studies with ribose-1-C^{14} in the normal subject have clearly demonstrated that the ribose escaping urinary excretion is rapidly metabolized, with prompt excretion of a portion of the first labeled carbon as CO_2. Since $C^{14}O_2$ appeared so soon after the injection of the labeled sugar, it is not likely that bacterial degradation of ribose that may have entered the gastrointestinal tract can be held responsible. Furthermore, the C^{14} ribose experiment in the diabetic has shown that the ribose not excreted in urine is largely converted to glucose by the sequence of reactions

of the pentose phosphate pathway as seen in Figure 1. Since ribose is converted to glucose, it appears probable that the $C^{14}O_2$ in expired air is derived from the oxidation of labeled glucose.

The phosphorylation of ribose to form ribose 5-phosphate appears to be a prerequisite step for ribose metabolism, and a D-ribokinase has been isolated from mammalian tissue (12). The question arises as to whether the serum phosphate drop after ribose infusion in man reflects this metabolic step. Since there seems to be no correlation between the dose of ribose and the phosphate fall, nor a difference in the phosphate decline after saline or ribose infusion (Table II, Subject J. D.), it appears unlikely that the observed phosphate changes have real significance. A fall in serum phosphate has been observed after the infusion of other pentoses (15).

Strangely enough, though ribose has been shown to be converted to glucose, hypoglycemia results after ribose infusion. This finding is apparently a species specific response, for Naito (7) reports that after the injection of ribose into the mouse and rabbit, there is an increase in the level of fermentable reducing substance, presumably glucose, in blood. A hypoglycemic response in man has been reported after the infusion of galactose into subjects with congenital galactosemia (40) and some normals (54), and although several laboratories report increases in blood glucose after fructose infusion (55, 56) one group of investigators has observed a hypoglycemic response to fructose (57). The production of hypoglycemia by ribose infusion is not an effect shared by xylose or other pentoses (15).

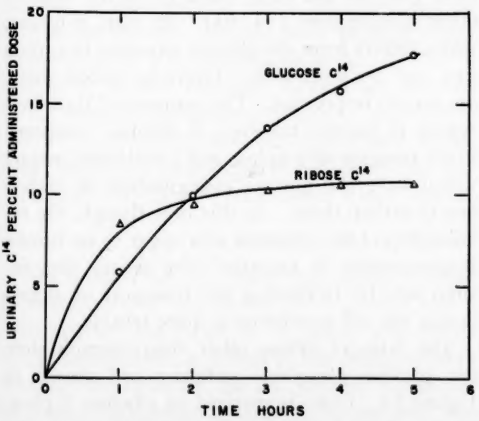

FIG. 13. URINARY EXCRETION OF C^{14} AS RIBOSE AND GLUCOSE AFTER INFUSION OF LABELED RIBOSE TO A DIABETIC SUBJECT

The lack of symptomatic response to ribose-induced hypoglycemia is of interest. Manifestations of hypoglycemia may be dependent on several factors, the rate of glucose fall, epinephrine release, and the low level of glucose actually encountered. It seems probable that the rate of fall in these experiments was sufficiently rapid and the glucose level low enough (15 to 30 mg. per cent) to cause symptoms. The lack of symptoms implies perhaps that neural tissue was able to utilize ribose or that sufficiently high glucose levels existed intracellularly. Indeed, Sable (58) has shown that ribose 5-phosphate is metabolized to hexose phosphate by brain homogenates. However, Park and co-workers (59) have demonstrated that ribose is unable to cross the blood-brain barrier in the rat, a fact which makes it doubtful that ribose itself could obviate a hypoglycemic response. In support of this is the fact that when insulin-induced hypoglycemia occurred, ribose did not appear to alleviate the symptoms.

Since the level of glucose in blood is dependent on the relative rates of entry and loss of glucose from blood, one has to consider the possibilities that ribose has either inhibited the hepatic mechanism determining the rate of entry of glucose into blood or increased the peripheral utilization of glucose via an insulin-like effect. It is conceivable that ribose causes a liberation of insulin from the pancreas. Hiatt (60) has obtained evidence that the hypoglycemic response to ribose in the dog is mediated by the pancreas. However, the observations we have made in man of a lack of blood phosphate and pyruvate change, associated with ribose-induced hypoglycemia, have led us to the belief that this effect of ribose may be primarily an hepatic one.

Under the conditions of our *in vitro* studies, the results indicate that an inhibition of phosphoglucomutase activity, causing a decrease in the rate of glycogen breakdown in the liver, could be responsible for the hypoglycemia reported here. Since we have only studied the ribose phosphate effect on one enzyme, we cannot state that this

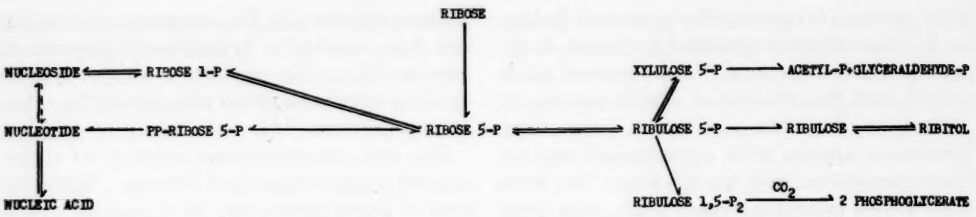

FIG. 14. FATES OF RIBOSE OTHER THAN ENTRANCE INTO THE PENTOSE PHOSPHATE PATHWAY OF METABOLISM

may be the sole locus of a possible defect in hepatic glucose output. In addition, it is only by conjecture and inference that we can extend observations made about an isolated enzyme to the intact cell or organism.

In the experiments with phosphoglucomutase *in vitro,* the direct addition of ribose ester caused an inhibition which could be ascribed to the presence of nonspecific organic phosphate (41). Therefore, it was necessary to demonstrate a more specific inhibition, and this was done with the preincubation type of experiment. In these studies, ribose 5-phosphate was used in excess of glucose 1-phosphate. However, since gram quantities of ribose are being metabolized in the *in vivo* experiments, it does not seem unreasonable that ribose 5-phosphate would be in excess of normally occurring amounts of glucose 1-phosphate. It is conceivable that the presence of significant amounts of intracellular ribose phosphate might well change the ionic environment of the mutase to produce the first type of inhibition. Possibly the ribose phosphate interacts with the enzyme and glucose 1,6-diphosphate according to Klenow and Emberland (43) to cause depletion of the latter cofactor and a state of dephosphorylation of the enzyme. This sequence would correspond to but not be identical to our preincubation period. It is interesting to point out that our *in vitro* observations parallel those observed of the effects of galactose 1-phosphate on the mutase reaction (38), that both sugars cause hypoglycemia, and that there has been shown an accumulation of intracellular galactose 1-phosphate in galactosemics (61), subjects who are known to have a hypoglycemic response to galactose infusion (40).

In both our *in vitro* studies and those reported with galactose 1-phosphate (38), isolated rabbit enzyme has been used. Though rabbits respond to ribose injection with a rise in blood glucose (7), this could be due to kinetic or other factors operating to rid the cell more quickly of ribose phosphate by conversion to glucose.

The observation of Levine, Goldstein, Huddlestun, and Klein (62) that insulin increases the volume of distribution of various sugars in the eviscerate dog has been corroborated in other animal species (59, 63). We have reported that in man, insulin causes an enhancement of the disappearance of the infused pentoses D-xylose, L-arabinose, and D-lyxose,, whereas D-arabinose is unaffected (16). Since the blood level of D-ribose is affected by insulin, this pentose may be grouped with the others similarly affected in the category of insulin responsive sugars. Although Goldstein, Henry, Huddlestun, and Levine (64) postulated a specificity of configuration for insulin responsive sugars, namely that carbon atoms one to three resembled the structure of these carbons in D-glucose, this has not been found to be the case by other investigators (54, 63). In man, D-lyxose, which differs from the glucose structure in carbon two, and D-ribose, which differs in carbon three, are insulin responsive. The response of these two sugars to insulin, however, is minimal compared to the response of D-xylose and L-arabinose, sugars which have the glucose configuration in carbon one to carbon three. At this time though, the relationship of the structure of a sugar to its insulin responsiveness is unsettled; the theory that insulin acts by facilitating the transport of sugars across the cell membrane is quite tenable.

The fates of ribose other than entrance into the pentose phosphate pathway are shown in Figure 14. Once isomerized to ribulose 5-phosphate, ribose 5-phosphate has several fates. An epimerization to xylulose 5-phosphate may ensue (65), whereupon a cleavage of the latter sugar

occurs (in bacteria) to form an acetyl phosphate and glyceraldehyde phosphate (66). Free ribulose has been reported to be present in normal human urine (67), suggesting that ribulose phosphate is dephosphorylated. Recently Hollmann and Touster (68) have demonstrated the presence of an enzyme in mammalian tissue, which is capable of reducing free ribulose to the sugar alcohol ribitol. Ribulose phosphate is also capable of being phosphorylated to ribulose 1,5-diphosphate (69) which acts as an important intermediate in the fixation of CO_2 in plants by forming two molecules of phosphoglycerate (70).

Ribose 5-phosphate may be changed to ribose 1-phosphate by the enzyme phosphoribomutase (71). Ribose 1-phosphate, interacting with purine bases under the influence of a nucleoside phosphorylase described by Kalckar (72), forms nucleosides which theoretically may be phosphorylated to yield a nucleotide, the structural component of nucleic acids. The main pathway for the formulation of nucleotides, however, involves the pyrophosphorylation of ribose 5-phosphate to form phosphoribosylpyrophosphate (73, 74). A summary of the reaction sequence of this pathway has recently been published by Wyngaarden (75).

According to Soskin and Levine (76), ribose forms a negligibly small part of the daily carbohydrate intake in man. It is ingested chiefly as a component of the nucleic acids of meat. Whether any part of this ingested ribose, or the ribose we infused, is incorporated intact into body nucleic acid is not known. Studies have suggested that ribose isolated from animals is synthesized from glucose or carbon fragments by enzymes involved in the pentose phosphate pathway of metabolism (77, 78). Recently, however, it has been shown that ribose may be incorporated directly into nucleic acid (79) in the HeLa cell in tissue culture.

SUMMARY

The metabolism of D-ribose was studied in man by the infusion of both labeled and unlabeled sugar. This pentose was found to be rapidly and extensively metabolized, a principal fate being conversion to body glucose via the pentose phosphate pathway. Although converted to glucose, the ribose infused caused a lowering of blood glucose, presumably by inhibiting the enzyme phosphoglucomutase, thus preventing glycogen breakdown in the liver. Ribose was found to be insulin responsive, although its response was smaller than that observed with other pentoses.

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THE INFLUENCE OF THE THYROID ON ADRENAL CORTICAL FUNCTION

By RALPH E. PETERSON

(From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md.)

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The evidence is quite conclusive from animal studies that an excess of circulating thyroid hormone results in adrenal enlargement, and hypothyroidism produced by surgical thyroidectomy or by administration of antithyroid drugs results in adrenal atrophy. This subject has been extensively reviewed by Money (1). Studies of adrenal size in patients with thyroid disease coming to autopsy have yielded conflicting reports. Means (2) reported no change in adrenal size in hyperthyroidism, Marine (3) and LeCompte (4) reported a decrease in size of the adrenal, and Holst (5) found enlarged adrenals in some patients with hyperthyroidism. Berkheiser (6) reported an advanced case of hypothyroidism, with atrophy of the adrenals and lipoid depletion.

Measurements of adrenal size may not, however, always represent a completely satisfactory "yardstick" for evaluation of adrenal cortical secretory activity (7, 8). In the past few years, several investigators have reported measurements of various plasma and urinary steroid fractions in hyper- and hypothyroidism in man. Several investigators have reported on the levels of free urinary corticoids (9–12) in hyperthyroidism, and in myxedema (9, 10). Because of the relatively nonspecific methods of assay used these data are difficult to interpret. Urinary 17-ketosteroids have usually been found to be low in both hyper- and hypothyroidism (9–18). Plasma 17,21-dihydroxy-20-ketosteroids have been reported to be normal in these diseases (19–21). Guinea pigs, which excrete a large fraction of the cortisol metabolites as free 17,21-dihydroxy-20-ketosteroids, show an increased excretion of free urinary corticoids after administration of thyroxine (20). In patients with clinical myxedema, the urinary level of the total free and glucuronide conjugated 17,21-dihydroxy-20-keteosteroids has been found to be decreased, whereas in hyperthyroidism the level of these urinary steroids is either normal or slightly increased (20).

Thyroidectomized rats (22) and cats (23) are reported to survive adrenalectomy better than animals with an intact thyroid, and administration of thyroid to the adrenalectomized cats produced sudden death of the animals. Also, in patients with Addison's disease, acute adrenal cortical failure may be precipitated if thyroid hormone is administered (24, 25). Primary myxedema has been observed in which the presenting findings were those of adrenal cortical insufficiency, and adrenal function returned to normal following thyroid hormone therapy (26).

With the advent of new and improved methods for the measurement of certain of the adrenal cortical hormones, and the availability of cortisol-4-C^{14}, it has become possible to further study the relationship of the thyroid hormone to adrenal cortical function. These techniques have been applied to a study of the rate of degradation and the rate of synthesis of cortisol in myxedema and thyrotoxicosis, before and after therapy. In this manner, much new information has been gained regarding the state of activity of the adrenal cortex in these diseases.

MATERIALS AND METHODS

Eight patients with the classical symptoms, signs, and laboratory findings of thyrotoxicosis (Grave's disease) and seven patients with findings typical of primary myxedema [1] served as the subjects of these studies. All the patients had hypo- or hyperthyroidism of moderate severity. Diagnoses were established on the basis of the

[1] The diagnosis of hypopituitarism would appear to have been ruled out on the basis of the following: *1*) normal plasma cortisol levels, *2*) return of the urine corticosteroid levels (A. A., M. H., G. B.) and cortisol turnover rates (M. H., H. A., A. A., G. B.) to normal following treatment with dessicated thyroid, and *3*) normal adrenal response to corticotropin in Patients W. C. and M. L.

clinical picture, basal metabolic rate, thyroid uptake of I^{131}, serum cholesterol, and serum protein-bound iodine. One normal subject was studied prior to and during the four week period following the administration of triiodothyronine (300 μg. per day) to induce a hyperthyroid state.

Plasma cortisol and urinary corticosteroids were determined by the modified (27, 28) procedure of Silber and Porter (29). Urinary 17-ketosteroids were determined by the Zimmermann procedure modified from the Holtorff and Koch method (30).

The steroids for intravenous administration were dissolved in ethanol and diluted to 300 or 500 ml. with 5 per cent dextrose in water to a final alcohol concentration of approximately 5 per cent. Following rapid infusion of the steroids, plasma samples were collected and assayed. The rate of disappearance of the steroids was expressed as a biologic half-time. Cortisol was assayed by the modified Silber-Porter procedure. The specificity of this assay procedure for cortisol in plasma following infusion of cortisol has been evaluated by isotope dilution (28) and found to be good. Cortisone and corticosterone were assayed by an isotope dilution method, since the phenylhydrazine assay for cortisone (31), and, to a lesser extent, the fluorometric assay for corticosterone (32), were not found to be specific for the determination of these steroids in plasma following their infusion. Urine cortisol concentration following the infusion of cortisol was also determined by the isotope dilution procedure.

The rapidly exchangeable miscible pool of cortisol and the rate of turnover of the pool [2] were determined by the previously described method (33). Two hundred to 300 μg. of cortisol-4-C^{14} (10 to 20 per cent of the pool size) were injected over a two to three minute period at 9:00 a.m. Plasma samples were collected every 30 minutes for the next three hours for measurement of the specific activity of cortisol.

RESULTS

Table I lists the plasma cortisol and urinary corticosteroid and 17-ketosteroid levels in the patients studied. The plasma cortisol level was found to be slightly elevated in one patient (G. B.) with untreated myxedema. In two patients with untreated hyperthyroidism, low values were found (D. Y., B. M.). In some of the subjects, determinations were made during the myxedematous or thyrotoxic period and again several months (3 to 12 months) following therapy; however, significant changes were not usually observed. The patients with myxedema were treated with desiccated thyroid (90 to 180 mg. per day). The patients with hyperthyroidism were treated with propylthiouracil (100 to 300 mg. per day), or with methimazole, except for Patient C. M., who received a therapeutic dose of radioactive iodine (4 mc.). D. B. was a normal subject made hyperthyroid by administration of triiodothyronine for four weeks.

All of the subjects with thyrotoxicosis had low levels of 17-ketosteroids in the urine. Normal subject D. B. showed a decline in excretion of 17-ketosteroids after the fourth week of therapy with triiodothyronine. All of the subjects with myxedema had a decreased urine 17-ketosteroids output, and the urine steroid level rose slightly following therapy with thyroid.

In the patients with thyrotoxicosis, the urinary corticoid levels were clearly elevated in D. Y. and B. M. In Patients B. D., C. M., and R. W. (females), the urinary corticoid levels were only slightly above the normal range of 4 to 10 mg. per day for females. No elevation of urinary corticoids occurred in the normal subject (D. B.) until the third week of treatment with triiodothyronine. All of the subjects with myxedema had urine corticoid levels below normal, and these returned to normal following therapy with thyroid.

Cortisol (100 to 200 mg.) was infused into eight subjects with thyrotoxicosis and five patients with myxedema (Table I). Figure 1 shows the curves of disappearance of cortisol from the plasma of a normal subject, and a patient with hyperthyroidism, and one with myxedema. In the hyperthyroid group, the infused cortisol was removed from the plasma at a greatly accelerated rate, except in the case of Patient V. H. This subject had a moderately enlarged liver and minimally abnormal liver function tests. In all but one of the patients (M. H.) with myxedema, there was a delayed disappearance of cortisol from the plasma. In both groups, following appropriate therapy for the thyroid disorder, the plasma disappearance rates of the infused cortisol returned toward the normal range.

The metabolisms of infused cortisone and corticosterone were found to be altered in the same direction as cortisol (Table I).

The urinary concentration of cortisol was determined in three patients (D. Y., R. W., and

[2] Turnover rate is defined as the quantity of newly synthesized cortisol added to the miscible pool, presumably by the adrenals, per unit time. The miscible pool is defined as that body of cortisol whose mixing is rapid relative to its turnover.

TABLE I

Plasma and urine steroid levels and biologic half-times of infused steroids

		Plasma cortisol μg. %	Urinary steroids mg./day		Biologic half-times* (hrs.)		
			Corticoids	17-Keto-steroids	F	E	B
Hyperthyroidism							
V. H. ♀	untreated	15			1.83	0.53	
E. S. ♀	untreated	11			1.0		0.67
B. D. ♀	untreated	8	12.0	5.0	0.92	0.27	
C. M. ♀	untreated	18	11.2	4.7	0.63		
	myxedema	15	2.6	3.3	2.15		
	treated	15	4.0	4.0			
D. Y. ♂	untreated	3	25.0		0.67		
	treated	13					
R. W. ♀	untreated	11	10.9	3.6	0.92		
W. C. ♂	untreated	13	7.5	7.5			
B. M. ♀	untreated	4	28.0	5.5	0.83	0.20	0.67
	treated	14	7.8	10.5	1.83		1.33
D. B. ♂	T-3†	8	22.0	15	1.16		
	control	22	12.0	25	1.83		
Myxedema							
N. F. ♀	untreated	21	2.5	1.8	3.25	0.92	
A. A. ♀	untreated	19	2.8	3.5	2.35		2.35
	treated	20	8.5	4.5	1.50		1.43
W. C. ♂	untreated	18	5.0	8.8			
M. H. ♀	untreated	11	2.5	3.0	1.83	0.60	
	treated	13	6.2	5.2			
M. L. ♀	untreated	9	2.2	2.3	2.60		
H. A. ♂	untreated	11	3.5	3.1			
G. B. ♀	untreated	28	2.0	2.5	2.70	0.58	1.83
	treated	19	7.0	5.4	1.58		
Normals							
	Range	6–25	♀ 4–10	♀ 6–15	1.50–2.00	0.38–0.58	1.16–1.50
	Mean, S. D.	14±3.5	6±1.2	10±1.0	1.83±0.10	0.45±0.03	1.33±0.05
			♂ 4–14	♂ 10–25			
			7±1.5	15±1.5			

* F = Cortisol; E = Cortisone; B = Corticosterone.
† T-3 = Triiodothyronine.

B. M.) with thyrotoxicosis following the infusion of 200 mg. of cortisol. In all subjects, the urine cortisol concentration (0.8, 1.0, and 2.0 mg. per 24 hours) was found to be lower than that obtained in five normal subjects given 200 mg. cortisol intravenously (2.5 to 3.8 mg. per 24 hours). In one patient with myxedema (M. L.), the urine cortisol concentration in the 24 hour urine obtained following the infusion of 200 mg. of cortisol was 5.6 mg.

Table II shows the data on the miscible pool of cortisol and its turnover rate in the patients with myxedema and thyrotoxicosis. For several of these patients data are also included for the period (3 to 12 months) following therapy for the thyroid disorder. In the normal subject (D. B.), the control studies were carried out prior to treatment with triiodothyronine. It may be noted that the fraction of the pool replaced per hour was much increased above normal in the hyperthyroid subjects and less than normal in the myxedema patients. The size of the miscible pool of cortisol in both the hyperthyroid and myxedema patients was not greatly different from that found in normal subjects. Also, in most of the patients studied before and after therapy the pool did not show a striking change in size. In Subject D. B., and Patients D. Y. and B. M. with hyperthyroidism, both the plasma cortisol and miscible pool of cortisol changed significantly in the same direction with a change to the euthyroid state.

In Table II the figures listed for milligrams of cortisol secreted per day may have much less significance than those listed for milligrams of corti-

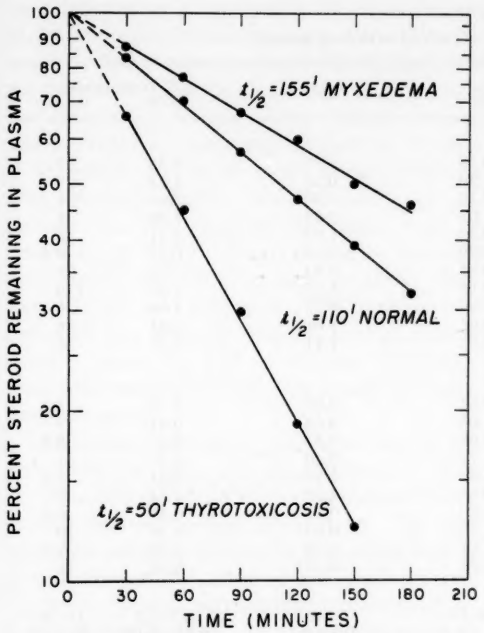

FIG. 1. DISAPPEARANCE OF CORTISOL FROM PLASMA FOLLOWING INTRAVENOUS ADMINISTRATION
Myxedema, Patient M. L.; thyrotoxicosis, Patient B. M.

sol secreted per hour. This results from the fact that the turnover rate studies cover only a three hour period of the day (9:00 a.m. to noon). These figures are, however, included for the benefit of those who may wish to compare them to the milligrams of urinary corticoids secreted per day in these same patients.

Figure 2 shows the specific activity curves of cortisol-4-C^{14} following the infusion of a trace quantity of labeled cortisol into Patient C. M.

DISCUSSION

The presence of normal plasma cortisol levels in most of the subjects with thyrotoxicosis or myxedema might at first suggest that there is no alteration in adrenal cortical secretion or rate of degradation of cortisol. The concentration of cortisol in the plasma is, however, not always a reliable index of the state of activity of the adrenal cortex since the level of cortisol in the plasma is maintained through the balance of the forces of production and destruction of the hormone.

The 24 hour urinary steroid assay is essentially a measure of metabolites of cortisol and is a reflection of the quantity of cortisol secreted during a day. This procedure represents, however, a rather crude index and suffers the following shortcomings: (*a*) except for a small fraction of free steroid metabolites, it measures only the fraction of steroid metabolites of cortisol excreted as steroids conjugated as glucuronides (*27*); (*b*) the steroid glucuronide conjugates in the urine normally make up only about 60 per cent of the metabolites of cortisol, but only about one-half of the 60 per cent retain the dihydroxy acetone side chain (*27*) and thus react with phenylhydrazine in sulfuric acid and ethanol to form the characteristic yellow chromophore absorbing maximally at 410 mμ; (*c*) a small but variable fraction of the phenylhydrazine reacting material may be nonsteroidal.

Disappearance of cortisol from the plasma is a reflection of the rate of catabolism of the hormone by the liver (*27*), since only a small quantity is excreted as unaltered cortisol. With but one exception, all of the patients with thyrotoxicosis stud-

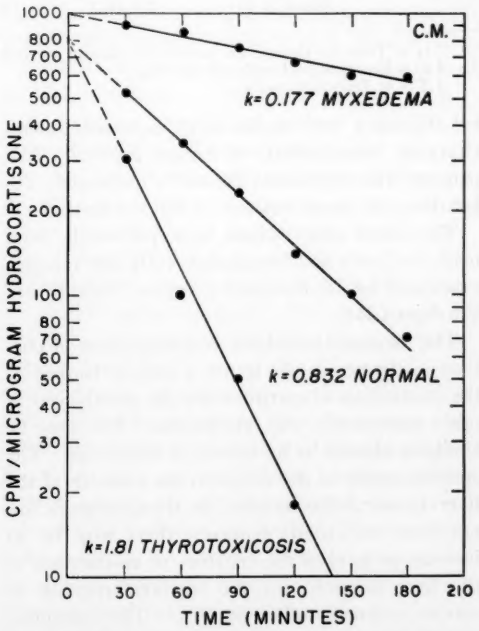

FIG. 2. PLASMA CORTISOL SPECIFIC ACTIVITY FOLLOWING INTRAVENOUS ADMINISTRATION OF CORTISOL-4-C^{14} TO PATIENT C. M. DURING PERIODS OF THYROTOXICOSIS, MYXEDEMA, AND THE EUTHYROID (NORMAL) STATE

TABLE II

Miscible pool and turnover rate of cortisol in thyroid disease

		Pool *mg.*	$t_{\frac{1}{2}}$* *hrs.*	k†	Turnover rate *mg./hr.*	*mg./day*
Hyperthyroid						
D. B.	T-3‡	1.2	0.38	1.82	2.26	55
	control	2.4	1.30	0.51	1.22	29
D. Y.	untreated	1.0	0.28	3.90	3.90	94
	treated	3.6	1.07	0.65	1.30	31
C. M.	untreated	1.3	0.38	1.82	2.41	58
	myxedema	1.2	3.92	0.18	0.22	5.3
	treated	1.2	0.83	0.83	1.02	24
B. M.	untreated	0.8	0.18	5.75	4.60	110
	treated	1.5	0.89	0.72	1.08	26
W. C.	untreated	1.8	0.67	1.04	1.91	46
R. W.	untreated	1.6	0.61	1.14	1.83	44
Myxedema						
M. H.	untreated	2.0	9.64	0.07	0.15	3.5
	treated	1.4	1.05	0.66	0.94	23
H. A.	untreated	1.1	2.08	0.33	0.37	8.8
	treated	3.3	1.77	0.40	1.30	31
A. A.	untreated	0.8	2.33	0.29	0.23	4.5
	treated	1.3	0.55	1.26	1.64	39
G. B.	untreated	2.4	4.60	0.15	0.36	8.6
	treated	2.0	1.10	0.63	1.25	30
M. L.	untreated	1.1	2.64	0.26	0.29	7.0
W. C.	untreated	2.2	2.75	0.25	0.55	14
Normals						
	Range	1.1–2.4	1.0–2.3	0.3–0.7	0.7–1.2	17–29
	Mean, S. D.	1.7±0.2	1.4±0.2	0.5±0.1	0.85±0.08	20±1.0

* $t_{\frac{1}{2}}$ = Time for the specific activity of plasma cortisol to be reduced by 50 per cent.
† k = Fraction pool replaced per hour.
‡ T-3 = Triiodothyronine.

ied showed a very significantly increased plasma clearance (metabolism) of infused cortisol. The patients with myxedema showed a moderately delayed rate of transformation of infused cortisol.

The above observations have previously been made by Levin and Daughaday (20) and recently confirmed by Di Raimondo, Sagan, Perloff, and Forsham (34).

The increased metabolic transformation of cortisol in thyrotoxicosis is not a unique feature of the metabolism of cortisol since the metabolism of other compounds, *viz.*, cholesterol (35), may be similarly altered by an excess of thyroxine. The specific nature of the defect in the capacity of the liver to metabolize cortisol in thyrotoxicosis has not been elucidated; however, there may be an increase in level of the enzymes or co-enzymes in the liver required for the biotransformation of cortisol and many other steroids.[3] The concentration of a number of oxidative enzymes is increased in the liver and other tissues of animals following the administration of thyroid, and thyroidectomy is usually followed by a decreased level of these same enzymes (37). The data obtained on the rate of metabolism of other steroids (cortisone, corticosterone) infused into patients with thyrotoxicosis suggests that there may be an increased level of many of the enzymes involved in the metabolic transformations of steroids (38).

With the aid of cortisol-4-C^{14} and utilization of conventional tracer techniques to study the miscible pool and turnover rate of cortisol, it has been possible to show that in patients with myxedema, the rate of synthesis of cortisol by the adrenals was decreased in the presence of a normal miscible pool. In thyrotoxicosis the turnover rate studies showed that the adrenals were producing an increased amount of cortisol in the presence of a normal miscible pool.

In thyrotoxicosis the increased production of cortisol in the presence of eucorticism was in many

[3] Crude liver homogenates from rats made thyrotoxic with thyroxine produce a twofold increase in the rate of degradation of cortisol when compared to liver homogenates from normal rats (36).

cases reflected by an increase in the concentration of cortisol metabolites in the urine. Patient W. C., however, had a normal urine corticoid level, yet the calculated value for daily cortisol production was about twice normal. Also, cortisol turnover was increased approximately twofold in Patients C. M. and R. W.; however, the urinary corticoid level was only slightly above normal. An explanation for this apparent discrepancy may be related to the unreliability of the urine corticoid assay as discussed previously.[4] Bradlow, Hellman, Zumoff, and Gallagher (39) have recently published data showing that the level of thyroid function alters the type of urinary metabolites from both endogenous and exogenously administered androgens.

The findings reported in this paper may be explained on the basis of a single primary defect, *i.e.*, alteration in rate of enzymatic transformation of the circulating cortisol by the liver. The liver may thus influence indirectly the production of cortisol through its control of one of the humerol regulators (plasma cortisol level) controlling secretion of corticotropin. In thyroid disease, as in liver disease, it may be the functional capacity of the liver to metabolize cortisol and not the requirements of the tissue cells that dictate the control of the pituitary adrenal cortical system.

An alternate hypothesis might be that thyroid hormone acts directly on the pituitary or adrenal to control cortisol secretion. In animals it has been found that an intact pituitary is necessary to obtain adrenal cortical hypertrophy following thyroid administration (40, 41). This observation in animals would also seem to rule out the possibility that the excess circulating thyroid hormone acts directly on the adrenal cortex independent of the pituitary (or circulating corticotropin) to increase cortisol secretion. If the thyroid hormone were to act directly on the pituitary or adrenal, it would be necessary to invoke more than a single defect since the liver is clearly involved and must be included in any explanation in order to account for the normal plasma cortisol level.

A renal mechanism probably need not be introduced to explain the reported observations since only a small fraction of the secreted cortisol is lost via the kidneys [50 to 150 μg. per day (42)]. Also, the data on the concentrations of cortisol in the urine after infusion of cortisol to normals and patients with thyroid disease demonstrate that very little of the circulating cortisol is disposed of by the kidneys.

In both the thyrotoxic and myxedematous patients, there was a low urinary output of neutral 17-ketosteroids. In the patients with thyrotoxicosis, the low 17-ketosteroids are probably not the result of a decreased conversion of 17-hydroxy steroids such as cortisol or its metabolites to 17-ketosteroids, since normally only a small fraction (about 10 per cent) of the cortisol is metabolized to 17-ketosteroids. Also, these patients actually had a greater quantity of cortisol available for transformation to 17-ketosteroids. The only explanation that is apparent at this time without additional data is that in thyrotoxicosis, the adrenal cortex secretes cortisol in preference to the androgens (17-ketosteroids).

The various clinical implications of these studies deserve some comment. The data would indicate that patients with myxedema have a relative adrenal insufficiency, especially as regards secretion of cortisol. Under normal or basal conditions, such patients experience no difficulty because they metabolize the secreted cortisol at a decreased rate. By virtue of a normally intact pituitary-adrenal cortical system, the patient with myxedema is not subjected to a plethora of cortisol. Under conditions of stress, the adrenals of these patients may be able to effect an adequate response, which, however, need only be subnormal since any additional cortisol secreted will remain for a longer time in the miscible pool. It is well known that administration of thyroxine to patients with Addison's disease may precipitate adrenal crisis (24,

[4] Following the injection of cortisol-4-C^{14} to patients with thyrotoxicosis and myxedema, an average of 77 per cent of the radioactivity was present in the first 24 hour urine collection. Glucuronidase hydrolysis and dichloromethane extraction of these same urines resulted in an average recovery of 45 per cent of the administered radioactivity. These data are similar to the data reported for normals (27), and demonstrate that in thyroid disease there is no alteration in either the total quantity of the cortisol metabolites excreted or that fraction conjugated as glucuronides. It is possible, as suggested by the studies of Levin and Daughaday (20), that in thyrotoxicosis there is a more extensive degradation of the steroid molecule; *viz.*, a preferential increased reduction of the C-20 ketone group of cortisol to form a larger fraction of nonphenylhydrazine-reacting urinary steroid metabolites.

25), and it might be anticipated that if a patient with severe myxedema were treated too vigorously with thyroid, adrenal cortical insufficiency could be induced.

The thyrotoxic individual is capable of manufacturing each day a quantity of cortisol that in a normal subject would quickly induce a state of hypercorticism. It might be postulated that if the hyperthyroid state were severe enough, as in a thyroid crisis, the rate of transformation of secreted cortisol might exceed the capacity of the adrenal to synthesize cortisol, and thus adrenal insufficiency might develop in the presence of an overproduction of cortisol. [In normal subjects, the adrenal appears to be capable of synthesizing 150 to 200 mg. of cortisol per day with maximal corticotropin stimulation (33).] Thus, such patients might be benefitted by exogenously administered cortisol and indeed this has been advocated as an important therapeutic adjunct to the treatment of the thyroid crisis (43).

SUMMARY

Plasma cortisol levels were normal in most patients with myxedema or thyrotoxicosis. Urine 17-ketosteroids were low in both myxedema and thyrotoxicosis. Urine corticoids were low in myxedema, and normal or moderately elevated in thyrotoxicosis. The urine corticoids returned to normal more promptly than the 17-ketosteroids following treatment of the thyroid disorder. Infused steroids were found to disappear from the plasma at an increased rate in thyrotoxicosis, and at a decreased rate in myxedema. Appropriate therapy for the thyroid disease returned the metabolism of the infused steroids to normal. The rate of synthesis of cortisol was reduced in myxedema and increased in thyrotoxicosis, and these alterations in the rate of secretion of cortisol were not associated with a significant change in the size of the miscible pool. Institution of the euthyroid state in these patients returned adrenal cortisol production to normal.

It is suggested from these data that there is a homeostatic mechanism mediated through the liver-pituitary-adrenals which results in a decreased synthesis of cortisol in patients with myxedema in whom the rate of removal of cortisol by the liver is impaired, and an increased synthesis of cortisol in patients with thyrotoxicosis in whom the rate of removal of cortisol by the liver is accelerated.

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Dr. Jacob Robbins of the National Institute of Arthritis and Metabolic Diseases made available several of the patients with thyroid disease used in this study.

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AN ANALYSIS OF BICARBONATE REABSORPTION DURING PARTIAL INHIBITION OF CARBONIC ANHYDRASE [1]

By WILLIAM B. SCHWARTZ,[2] ADRIEN FALBRIARD,[3] AND ARNOLD S. RELMAN

(From the Department of Medicine, Tufts University School of Medicine, and Boston University School of Medicine, and from the Medical Service of the New England Center Hospital and the Evans Memorial, Massachusetts Memorial Hospitals, Boston, Mass.)

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A considerable body of evidence supports the view that the bicarbonate diuresis produced by acetazolamide (Diamox®) results from inhibition of carbonic anhydrase activity in renal tubular cells (1–3). In a preliminary study (4), it was found that this diuresis was reduced if metabolic acidosis was induced prior to the administration of the drug. This phenomenon has subsequently been observed repeatedly in clinical and experimental studies (5–8). It is now well known that patients with metabolic acidosis do not respond to acetazolamide and that the acidosis resulting from the administration of acetazolamide is an important factor in the development of resistance to continued treatment with this drug (9–11).

Some workers have found an apparent threshold for acetazolamide activity at plasma bicarbonate levels of approximately 16 mEq. per L. (6). At levels below this threshold, oral administration of acetazolamide is said to have no effect. Other studies have reported marked reduction of acetazolamide action in metabolic acidosis but no absolute threshold (5).

The explanations given for the reduction in response during metabolic acidosis are that *a*) a certain portion of the bicarbonate reabsorptive process in the renal tubule is normally not dependent on carbonic anhydrase activity, or that *b*) the importance of the enzyme in the reabsorptive process is in some way reduced by metabolic acidosis.

In the present experiments the action of acetazolamide in dogs was studied at several dose levels and during varying degrees of metabolic acidosis. With each of the doses separate but similar curves were obtained demonstrating a diminishing effect of the drug on the reabsorptive process as plasma bicarbonate concentration was reduced. The two larger doses of acetazolamide reduced reabsorptive rates significantly even at the lowest plasma levels studied, thus suggesting that carbonic anhydrase activity is involved in the reabsorption of most, if not all, of the filtered bicarbonate. An attempt has been made to explain these findings in the light of classical Michaelis-Menten enzyme kinetics.

MATERIAL AND METHODS

Sixty-three experiments were performed on 47 female mongrel dogs weighing from 9 to 15 Kg. Light general anesthesia was obtained by intravenous administration of morphine or sodium pentobarbital. Metabolic acidosis and the desired plasma levels of bicarbonate (5 to 22 mEq. per L.) were achieved by infusion of appropriate amounts of HCl or by oral administration of NH_4Cl. This was followed by continuous infusion of isotonic NaCl at a steady rate of 6.5 to 10 ml. per minute through a Bowman infusion pump.

Urine was collected under neutral mineral oil through an inlying bladder catheter and the bladder was emptied by manual compression at the end of each collection period.

The clearance of exogenous creatinine was used as a measure of glomerular filtration rate. Plasma and urine were analyzed for creatinine by a modification of the method of Bonsnes and Taussky (12) and for total CO_2 content by the manometric method of Van Slyke. Blood and urine pH were determined anaerobically at room temperature in a syringe-type Cambridge pH meter and corrected to 37° C. (using a correction factor of 0.01 units per degree C.). Carbon dioxide tension in urine and blood was calculated from the Henderson-Hasselbalch equation, using a value of 6.1 for the pK_1 and a value for α in blood of 0.0301 and in urine of 0.0309. Bicarbonate concentration was calculated as the difference be-

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TABLE I

Comparative effects of 1 mg., 5 mg., and 20 mg. per Kg. of acetazolamide on bicarbonate excretion and reabsorption at similar plasma bicarbonate concentrations *

Time *min.*	Plasma pCO_2 *mm.Hg*	Plasma HCO_3 *mEq./L.*	Urine pH	Urine HCO_3 filt. *μEq./min.*	Urine HCO_3 excret. *μEq./min.*	Urine HCO_3 reabs. *mEq./100 ml.* glomerular filtrate	GFR† *ml./min.*
Dog No. 165	Constant infusion of saline and creatinine						
0–10	40	13.9	5.07	1.357	1	1.46	93
10–20	40	14.3	5.07	1,470	1	1.50	98
20–22	Acetazolamide I.V. 20 mg./Kg.						
23–33	33	13.4	7.29	1,142	503	0.79	81
33–43	32	13.0	7.29	1,055	434	0.81	77
Dog No. 224	Constant infusion of saline and creatinine						
0–10	44	14.6	5.61	704	2	1.53	46
10–20	45	14.6	5.70	689	2	1.53	45
20–22	Acetazolamide I.V. 5 mg./Kg.						
23–31	37	14.2	7.25	581	133	1.15	39
31–39	37	13.8	7.17	595	116	1.17	41
Dog No. 190	Constant infusion of saline and creatinine						
0–10	38	13.9	6.52	1,153	26	1.43	79
10–20	39	14.0	6.57	1,176	27	1.44	80
20–22	Acetazolamide I.V. 1 mg./Kg.						
23–31	39	14.1	7.22	1,169	172	1.26	79
31–39	38	13.8	7.09	1,117	111	1.31	77

* A Donnan factor of 1.05 was used in calculating filtered load.
† Glomerular filtration rate.

TABLE II

Effects of 20 mg. and 5 mg. per Kg. of acetazolamide on bicarbonate excretion and reabsorption at low plasma bicarbonate concentrations *

Time *min.*	Plasma pCO_2 *mm. Hg*	Plasma HCO_3 *mEq./L.*	Urine pH	Urine HCO_3 filt. *μEq./min.*	Urine HCO_3 excret. *μEq./min.*	Urine HCO_3 reabs. *mEq./100 ml.* glomerular filtrate	GFR† *ml./min.*
Dog No. 233	Constant infusion of saline and creatinine						
0–10	33	7.1	5.18	368	1	0.75	49
10–20	34	7.6	5.39	344	1	0.80	43
20–21	Acetazolamide I.V. 20 mg./Kg.						
22–30	34	7.7	6.67	429	79	0.66	53
30–38	36	7.9	6.58	382	61	0.70	46
Dog No. 164	Constant infusion of saline and creatinine						
0–10	33	10.2	6.04	717	16	1.05	67
10–20	33	10.2	6.07	717	13	1.05	67
20–22	Acetazolamide I.V. 20 mg./Kg.						
23–33	27	9.1	6.97	566	222	0.58	59
33–43	28	9.3	6.94	608	182	0.69	62
Dog No. 223	Constant infusion of saline and creatinine						
0–10	30	9.7	5.82	755	3	1.02	74
10–20	30	10.5	5.84	781	3	1.10	71
20–22	Acetazolamide I.V. 5 mg./Kg.						
23–31	29	10.4	7.31	730	141	0.88	67
31–39	31	10.3	7.14	691	105	0.92	64

* A Donnan factor of 1.05 was used in calculating filtered load.
† Glomerular filtration rate.

tween the total CO_2 content and the dissolved carbon dioxide. Filtered bicarbonate was taken as the product of the filtration rate and the plasma bicarbonate concentration, corrected for a Donnan factor of 1.05.

Following at least two control periods of 8 to 10 minutes each, the acetazolamide was injected intravenously over a period of one to two minutes. One minute after the end of the injection, two or three more urine periods of 6 to 10 minutes each were obtained. Three dosages of acetazolamide were used: one mg. per Kg. of body weight, 5 mg. per Kg., and 20 to 40 mg. per Kg. Since no difference of action was observed between 20 mg. per Kg. and 40 mg. per Kg., this dosage range was assumed to achieve virtually the same degree of carbonic anhydrase inhibition. The acetazolamide solution was prepared by adding the powder to approximately 20 ml. of distilled water containing approximately 0.7 to 0.8 mEq. of alkali per 100 mg. of drug.

RESULTS

In every experiment with doses of 5 or 20 to 40 mg. per Kg., acetazolamide produced a definite increase in bicarbonate excretion. The magnitude of this effect diminished with increasing metabolic acidosis, but remained significant even at the lowest plasma bicarbonate levels. On the other hand,

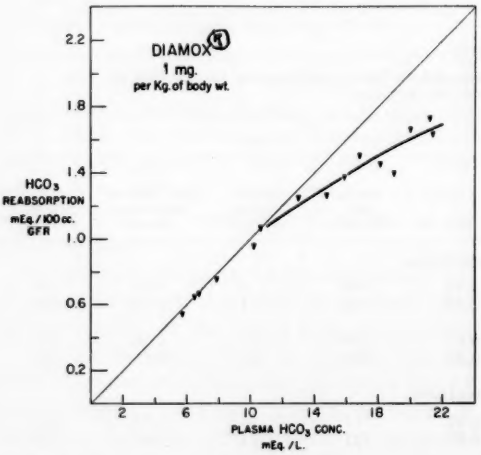

FIG. 1. RELATIONSHIP BETWEEN BICARBONATE REABSORPTION AND PLASMA BICARBONATE CONCENTRATION FOLLOWING ADMINISTRATION OF INTRAVENOUS ACETAZOLAMIDE 1 MG. PER KG.

Each point represents one experiment. The heavy line was drawn through the experimental points by inspection. The thin line bisecting the graph represents the control reabsorption. In this and all subsequent figures the plasma levels have been corrected by a Donnan factor of 1.05.

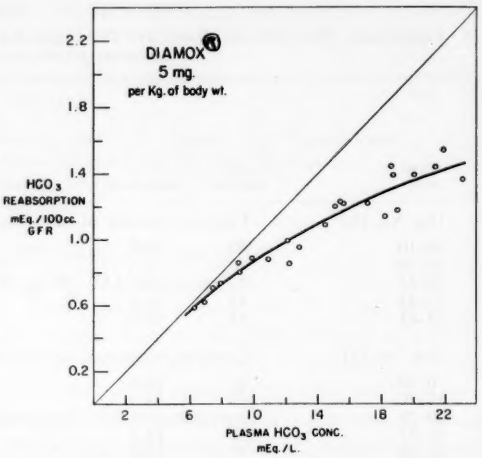

FIG. 2. RELATIONSHIP BETWEEN BICARBONATE REABSORPTION AND PLASMA BICARBONATE CONCENTRATION FOLLOWING ADMINISTRATION OF INTRAVENOUS ACETAZOLAMIDE 5 MG. PER KG.

See Figure 1 for details.

at any given plasma concentration, the higher the dosage of the drug the greater was the response. With 1 mg. per Kg. a significant effect on bicarbonate excretion was observed at the higher plasma bicarbonate levels, but below 12 mEq. per L. the effects were negligible. Table I compares the effect of 20 mg., 5 mg., and 1 mg. per Kg. of acetazolamide on bicarbonate excretion and reabsorption at a plasma bicarbonate level of approximately 14 mEq. per L. It is apparent that the effect on bicarbonate reabsorption increased with the dose. That an effect could be clearly demonstrated even at low plasma levels is illustrated by Table II, which summarizes the results of three experiments at plasma bicarbonate levels of 8 to 10 mEq. per L. In the first two experiments, a dose of 20 mg. per Kg. was used; in the other experiment, the dose was 5 mg. per Kg. It is clear that the administration of the drug was followed in each instance by a definite effect on both excretion and reabsorption.

The relationship between the plasma bicarbonate level and the action of different doses of acetazolamide on bicarbonate reabsorption is summarized by Figures 1 to 3, which include all the experiments. On the abscissa of each graph is plotted plasma bicarbonate concentration and on

the ordinate is the minimum bicarbonate reabsorption following acetazolamide injection, expressed as milliequivalents of bicarbonate per 100 milliliters of glomerular filtrate.[4] This minimum reabsorption usually occurred in the first collection period following the injection. The curves have been drawn through the points by inspection. The thin straight line bisecting each plot indicates reabsorption during the control period, when there is essentially complete removal of the filtered bicarbonate. Thus the vertical distance between this line and each experimental point represents the magnitude of acetazolamide effect at each plasma level.

The first fact apparent from these curves is that acetazolamide action diminishes as the plasma bicarbonate level is reduced.

A second fact is that the different dosages of acetazolamide produce different curves of similar

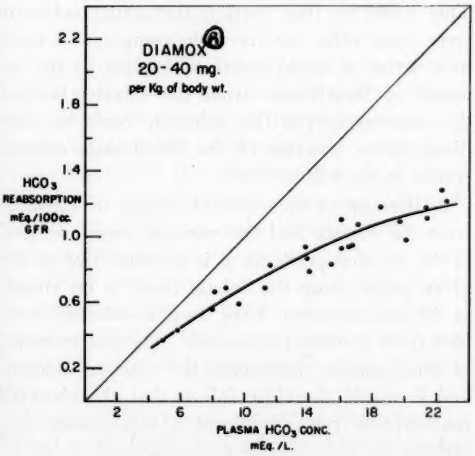

FIG. 3. RELATIONSHIP BETWEEN BICARBONATE REABSORPTION AND PLASMA BICARBONATE CONCENTRATION FOLLOWING ADMINISTRATION OF INTRAVENOUS ACETAZOLAMIDE 20 TO 40 MG. PER KG.

See Figure 1 for details.

[4] Correction of the reabsorption rates to a standard glomerular filtration rate is assumed to correct for differences in renal mass between different animals. In individual animals the changes in glomerular filtration rate following the injection of acetazolamide were usually negligible. The mean per cent change from the average control rate, without regard to sign, was 7.9 ± 5.5, 8.0 ± 6.1, and 8.5 ± 8.0 for the 1, 5, and 20 to 40 mg. experiments, respectively.

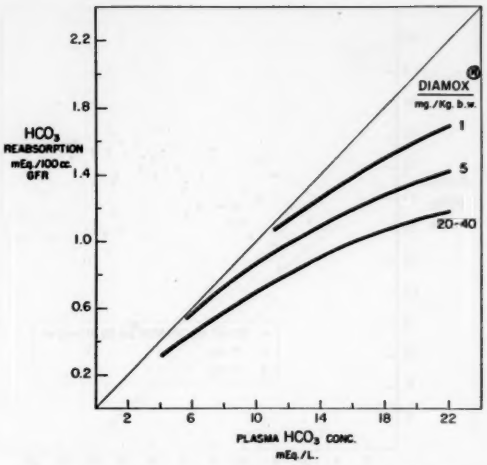

FIG. 4. RELATIONSHIP BETWEEN BICARBONATE REABSORPTION AND PLASMA BICARBONATE CONCENTRATION WITH THREE DIFFERENT DOSES OF ACETAZOLAMIDE

The heavy lines from Figures 1 to 3 are plotted together on this graph.

shape. This is illustrated by Figure 4 which compares the action of 1 mg., 5 mg., and 20 to 40 mg. of acetazolamide per Kg. of body weight on bicarbonate reabsorption.

It can also be seen that at plasma bicarbonate levels below 12 mEq. per L., 1 mg. per Kg. seems to have no significant effect on reabsorption.

Plasma pCO_2 was generally lower during severe acidosis, but respiratory compensation was negligible except with very severe acidosis, probably because of the anesthesia. Figure 5 shows the scatter of the plasma pCO_2 values in relation to plasma bicarbonate levels. There is a slight tendency for the pCO_2 values to fall with increasing acidosis, but this trend produces appreciable reductions in pCO_2 only at plasma bicarbonate levels below 12 mEq. per L. At each plasma bicarbonate level there is a wide and apparently random scatter of pCO_2 values with respect to acetazolamide dosage. No significant changes in plasma pCO_2 were produced by administration of acetazolamide.

DISCUSSION

The data summarized in Figure 4 show that with three different doses of acetazolamide and over a wide range of plasma bicarbonate concen-

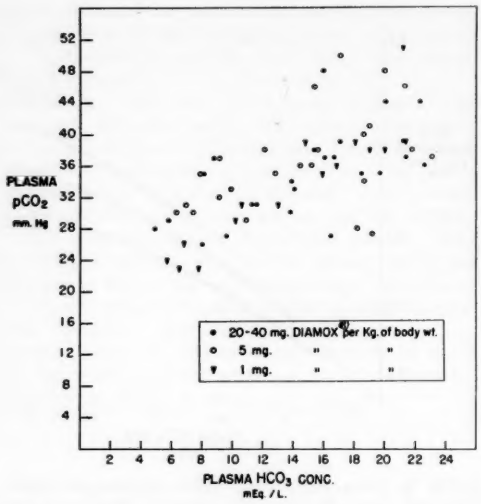

FIG. 5. RELATIONSHIP BETWEEN PLASMA CO_2 TENSION AND BICARBONATE CONCENTRATION FOR ALL EXPERIMENTS

Each point represents one experiment.

tration there is a progressively greater inhibitory effect on bicarbonate reabsorption. With doses of 5 and 20 to 40 mg. per Kg. acetazolamide significantly affects bicarbonate reabsorption even at plasma bicarbonate levels as low as 5 to 10 mEq. per L. The effect of 1 mg. per Kg. becomes negligible at a bicarbonate concentration of about 12 mEq. per L. If the only action of acetazolamide, at the doses used, is to partially inhibit carbonic anhydrase activity, it would seem to follow from these observations that carbonic anhydrase is normally involved in the reabsorptive mechanism for most of the filtered bicarbonate. Stated differently, it would appear that there can be only a small fraction of the filtered bicarbonate which is reabsorbed without the participation of the enzyme. If the above considerations are valid then the reabsorptive rates shown in Figures 1 to 4 would seem to be a function of residual enzyme activity after partial inhibition by acetazolamide.

No obvious explanation of the relationship between plasma bicarbonate concentration and bicarbonate reabsorption is at hand, but a tentative hypothesis may be offered. Assuming that the reabsorption of bicarbonate after administration of acetazolamide is dependent on enzyme activity, then the fact that three different curves are obtained with three different doses of inhibitor would suggest that in each instance the enzymatic reaction is the rate-limiting step in the overall process leading to bicarbonate reabsorption. If this were so, classical Michaelis-Menten enzyme kinetics might provide an approximate overall description of the reabsorptive process. To test the applicability of the Michaelis-Menten analysis in this situation, however, it is necessary to make several assumptions which would permit the use of the available data as terms in the kinetic equation. It is recognized that the validity of these assumptions may be questioned, but it is considered of interest to determine whether a unifying theory might be derived which could provide an adequate explanation for the data. For this purpose the following assumptions were made:

1. The bicarbonate in the tubular fluid is considered to be in effect the substrate for the enzyme. This could be true even if the actual substrate were some other intermediate compound formed in a series of rapid reactions leading to the removal of bicarbonate from the filtrate, because the concentration of this substrate could be some direct linear function of the bicarbonate concentration in the tubular fluid.

2. Because of the constant supply of substrate from the filtrate and the constant rapid removal of the reaction products, it is assumed that at any given point along the tubule there is no change in the concentration of the enzyme-substrate complex from moment to moment. The concentration of this complex determines the reaction velocity, and it would therefore follow that the observed reabsorption rates represent a steady-state condition.

3. In this application of the Michaelis-Menten analysis to the tubule as a whole, it has been assumed, finally, that the filtrate bicarbonate concentration is equal to, or is some direct and constant function of, the mean tubular bicarbonate concentration for the overall reabsorptive process.[5]

[5] One mechanism which might nearly satisfy the conditions of this assumption is suggested by micropuncture studies in Necturus. These indicate that the bicarbonate concentration remains constant throughout the proximal tubule (13). In the dog, this would mean that at least 80 per cent of the reabsorptive process occurs at a constant concentration. Obviously, if the urine is alkalinized

In the replotting of the experimental data, the Lineweaver-Burk modification of the Michaelis-Menten equation was used:

$$1) \quad v = \frac{V_{max}\, s}{K_m + s} \qquad \text{(Michaelis-Menten)}$$

$$2) \quad \frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} (1/s) \qquad \text{(Lineweaver-Burk),}$$

where

v = The steady-state velocity of the reaction (bicarbonate reabsorption per 100 ml. glomerular filtrate)

s = The substrate concentration (bicarbonate concentration per 100 ml. of filtrate)

V_{max} = The maximum velocity of the reaction (a constant for any given amount of enzyme)

K_m = The Michaelis constant for the enzyme, which has the dimension of a concentration.

Figure 6 shows the experimental data replotted according to equation *2*) with 1/v as the ordinate and 1/s as the abscissa. In this figure the six lowest points with 1 mg. per Kg. are omitted, since (Figure 1) they do not differ significantly from the straight line representing complete reabsorption, and since Michaelis-Menten kinetics only apply to situations in which enzyme is rate-limiting. With only 1 mg. per Kg. of inhibitor there apparently is enough residual enzyme activity to make the substrate the limiting factor when bicarbonate concentration drops below 10 to 12 mEq. per L.

The regression equations for the three lines indicated on the figure were calculated by the method of least squares. For 1 mg. per Kg. the equation is: $y = 0.26 + 0.74x$ ($r = 0.91$); for 5 mg. per Kg. it is: $y = 0.32 + 0.85x$ ($r = 0.98$); and for 20 to 40 mg. per Kg., $y = 0.33 + 1.14x$ ($r = 0.98$). It is apparent that each set of experimental points closely fits the straight line predicted by equation *2*). The slope of the lines increases with the dose of inhibitor used. This type of plot, with

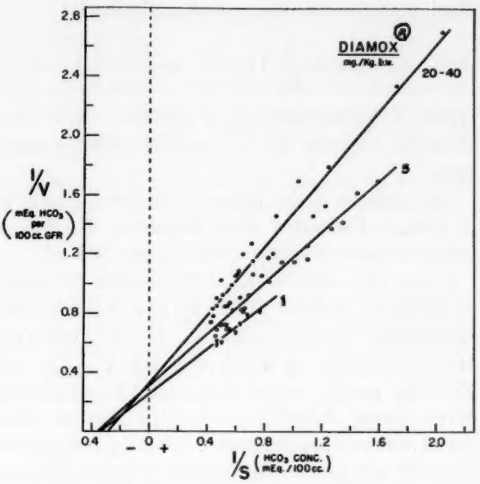

FIG. 6. RELATIONSHIP BETWEEN THE RECIPROCAL OF THE BICARBONATE REABSORPTION RATE AND THE RECIPROCAL OF THE PLASMA BICARBONATE CONCENTRATION (LINEWEAVER-BURK MODIFICATION OF THE MICHAELIS-MENTEN EQUATION)

The lines through each of the three sets of data were calculated by the method of least squares.

increasing doses of acetazolamide yielding distinct straight lines of increasing slope, is characteristic of the effect of an enzyme inhibitor on the rate of an enzyme-limited process.

Intersection of the lines on the abscissa is characteristic of noncompetitive inhibition, while intersection on the ordinate is typical of competitive inhibition. In Figure 6 it is apparent that the three intercepts on both axes are so close together that it is impossible to distinguish between these two types of inhibition. Recent *in vitro* studies indicate that sulfanilamide is a noncompetitive inhibitor of carbonic anhydrase (14), but the present data do not permit any firm conclusion about the exact type of inhibition produced by acetazolamide *in vivo*.

Different values of V_{max} for the uninhibited system can be calculated from the present data, depending upon the type of inhibition assumed. The calculation is made below for both types:

In noncompetitive inhibition, K_m is unaffected by the presence of the inhibitor, but V_{max} is decreased by a factor which is proportional to the concentration of the inhibitor. The Lineweaver-

or acidified in the distal fifth of the tubule, this would result in some discrepancy between the filtrate concentration and the actual mean. For the purpose of this analysis, it is assumed that any such discrepancy would not be critical.

Burk equation for this type of inhibition is:

$$3) \quad \frac{1}{v} = \left(1 + \frac{I}{K_i}\right)\left(\frac{1}{V_{max}} + \frac{K_m}{V_{max}} \cdot \frac{1}{s}\right),$$

where I = Concentration of inhibitor, K_i = Dissociation constant for the enzyme-inhibitor complex.

In equation 3, the intercept on the 1/s axis is $-1/K_m$. Estimated from Figure 6, K_m has a value of approximately 2.9 mEq. per 100 ml.

From this value of K_m it is possible to obtain a minimum estimate of the *in vivo* V_{max} for the uninhibited enzyme, since K_m is the concentration of substrate at which v equals $V_{max}/2$. At 29 mEq. per L., or for that matter at all plasma levels above 25 mEq. per L., the normal reabsorptive rate is approximately 2.5 mEq. per minute per 100 ml. glomerular filtrate (1). It follows that the value of V_{max} for the uninhibted enzyme *in vivo* would be at least 5.0 mEq. per 100 ml. glomerular filtrate.

On the other hand, if Figure 6 is interpreted as indicating competitive inhibition, V_{max} can be calculated from the Lineweaver-Burk equation for this type of inhibition:

$$4) \quad \frac{1}{v} = \frac{1}{V_{max}}\left(K_m + \frac{K_m(I)}{K_i}\right)\frac{1}{(s)} + \frac{1}{V_{max}}.$$

Estimated as the reciprocal of the intercept on the y axis, the mean value of V_{max} calculated from the regression equations of Figure 6 would thus be 3.4 mEq. per 100 ml. glomerular filtrate.

Assuming either type of inhibition, the data thus suggest that the normal maximal rate of bicarbonate reabsorption of approximately 2.5 mEq. per 100 ml. glomerular filtrate is less than the maximal rate at which the carbonic anhydrase reaction could proceed. It is, therefore, tentatively proposed that the kinetic limitations of this enzyme are not responsible for the establishment of the normal bicarbonate threshold.

The data are consistent with the hypothesis that at plasma bicarbonate levels below the usual threshold, partial inhibition of carbonic anhydrase makes enzyme activity the rate-limiting step in the process of bicarbonate reabsorption. Under these conditions the overall process of reabsorption appears to follow the Michaelis-Menten relationship in a consistent fashion. It is evident that normally, in the absence of an inhibitor, the reabsorptive process is virtually complete at all plasma levels below 25 mEq. per L. In other words, substrate presentation (filtered bicarbonate) and the velocity of the reaction (bicarbonate reabsorption) bear a linear relationship to each other at subthreshold levels. This would suggest that in the absence of an inhibitor the enzymatic reaction is not the rate-limiting step. Some other process, in all likelihood the filtration of bicarbonate, probably limits the rate of presentation of substrate to the enzyme.

While the behavior of the system under study apparently can be described in terms of enzyme kinetics, it is obvious that many other factors might influence it. Changes in cellular potassium content as well as marked alterations in pCO_2 are known to affect bicarbonate reabsorption. The mechanisms through which these influences operate are not clarified by this study, but the present observations may provide a reasonable framework for further investigations of these problems.

SUMMARY

The effects on renal bicarbonate reabsorption of 1, 5, and 20 to 40 mg. per Kg. of acetazolamide have been studied in acidotic dogs whose plasma bicarbonate concentrations ranged from 5 to 22 mEq. per L. With each of the doses separate but similar curves were obtained demonstrating a diminishing effect of the drug on the reabsorptive process as plasma bicarbonate concentration was reduced. The two larger doses of acetazolamide reduced reabsorptive rates significantly even at the lowest plasma levels studied, thus suggesting that carbonic anhydrase activity is involved in the reabsorption of most, if not all, of the filtered bicarbonate. An attempt has been made to explain these findings in the light of classical Michaelis-Menten enzyme kinetics.

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COMPARATIVE STUDY OF GLUCOSE AND FRUCTOSE METABOLISM IN INFANTS WITH REFERENCE TO UTILIZATION AND TO THE ACCUMULATION OF GLYCOLYTIC INTERMEDIATES [1]

By ROBERT KAYE, MARGARET L. WILLIAMS, AND GIULIO BARBERO

(From the Children's Hospital of Philadelphia, and the Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, Pa.)

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A number of studies of the comparative utilization [2] of intravenously administered glucose and fructose have been carried out in adults (1–4). The results have almost uniformly favored fructose, but have been concerned for the most part with infusions which were of short duration and quantitatively inadequate from the standpoint of caloric maintenance. The caloric equivalents of the differences in the excretion of the sugars were clinically insignificant.

Comparative utilization studies involving amounts of carbohydrate adequate for the provision of caloric maintenance have been carried out by Strub, Best, Consolazio, and Grossman (4) and by Jonxis and Huisman (5). The former group administered fructose and invert sugar (equal parts of glucose and fructose) in 300 Gm. quantities over a period of three hours and observed approximately 95 per cent retention in both cases. Jonxis and Huisman, who carried out the only study previously reported in children, were unable to elicit any difference in utilization of glucose and invert sugar. However, the rate of carbohydrate administration employed in their experiments, which were of 24 hours duration, was sufficiently slow to permit essentially complete utilization of both sugars.

In conflict with the consensus of a large number of studies, Beal, Smith, and Frost (6) have found fructose losses in the urine to exceed those of glucose. These authors have explained the discrepancy between their results and those of others on the basis of a delayed excretion of fructose following completion of the infusion and return of the blood level to normal.

The importance of parenteral alimentation in infants suggested that a comparative study of the utilization of glucose and fructose in this age group would be of value. The present study was designed to carry out the comparison at a level of intake which would provide sufficient calories for caloric maintenance.

The rates of administration were selected *a*) to determine whether fructose utilization equals the maximum generally considered to obtain for glucose (1 Gm. per Kg. per hour) (7) and *b*) to compare utilization of the two sugars when administered at rates in excess of the infants' capacity for relatively complete glucose utilization (2 Gm. per Kg. per hour).

The appearance of certain disadvantageous side effects during the course of fructose infusions at the more rapid rate, and several reports from other laboratories of the development of acidosis with fructose administration (8, 9) lead to investigation of the attendant acid-base changes. A further study was carried out to compare the extent of accumulation of certain acid metabolites and the magnitude of the acid-base disturbance which results from the rapid infusion of glucose and fructose. The data derived from this study have bearing on the clinical use of these two carbohydrates.

MATERIALS AND METHODS

Studies on utilization. Eleven male infants convalescent from neurosurgical procedures were used as subjects. Their average age was 6.4 months (range, 2 to 16 months) and their weight averaged 7.2 Kg., (range, 5.4 to 10 Kg.). They were divided into two groups. Group A consisted of three individuals given infusions of each of the carbohydrates at a rate of approximately 1.0 Gm. per Kg. per hour. Group B consisted of eight subjects who received a total of 17 hexose infusions at a rate

[1] This work was supported by grants from the National Institutes of Health and the Mead Johnson Company.

[2] The term "utilized" is used to denote the difference between carbohydrate input and renal excretion during the duration of the study period.

TABLE I

Composition of solutions

		Fructose			Glucose		
Hexose	Gm./100 ml.	5.0	7.2	10.1	5.1	7.2	10.1
Na	mEq./L.	33.5	33.5	33.0	33.5	33.0	32.5
K	mEq./L.	17.0	16.7	16.7	17.4	17.2	17.0
Cl	mEq./L.	42.7	44.4	44.4	44.4	42.7	42.7
Phosphate	mM/L.	5.9	6.0	6.0	5.0	6.0	5.9
pH		4.30	4.30	4.30	4.35	4.45	4.45

of 2.0 Gm. per Kg. per hour. With the exception of one subject who received three infusions, one of glucose and two of fructose, all were given one infusion of each of the carbohydrates, with the initial carbohydrate randomized. The glucose and fructose were infused on different days with the subject in equivalently good states of hydration on both days.

The solutions were administered at a rate of approximately 25 ml. per Kg. per hour for a period of six hours except for the experiments on Subjects 3 and 10, which were of four hours duration. A positive pressure pump was used to secure a uniform rate of administration. The solutions, whose compositions are given in Table I, were prepared by the Mead Johnson Research Laboratories. They were designed to provide constant amounts of water and electrolytes and increasing hexose concentrations of 5.0, 7.15, and 10.0 Gm. per 100 ml.

Observations of pulse, respiratory rate, and liver size were made at intervals of two hours.

Analyses of the blood were carried out prior to the infusion for the hematocrit and concentrations of glucose and fructose, sodium and potassium. Carbohydrate analyses were repeated at intervals of two hours during the infusion and one hour after its completion. Final determinations of sodium and potassium and of the hematocrit were performed at the end of the infusion. Glucose and hematocrit determinations were done on capillary blood. The other analyses were done on serum obtained from venous blood. The methods used were the Nelson modification of the Somogyi method for total reducing substance (10), Schreiner's modification of the Roe method for fructose (11), and flame photometry for sodium and potassium.

Complete urine collections were obtained. Separate analyses were carried out on aliquots of the collections at two hourly intervals during the infusion, at one hour postinfusion and finally for an additional four to eight hours. Urine was analyzed for glucose and fructose, sodium, potassium and specific gravity.

The quantity of carbohydrate excreted in the urine following the first postinfusion hour was negligible in all cases and was therefore omitted from the calculations. This finding is at variance with the observations of Beal, Smith, and Frost (6).

Study of acid-base changes. The experimental group here consisted of 15 hospital in-patients who were convalescent from a variety of diseases. They ranged in age from 11 weeks to 2 years and in weight from 5.3 to 12.7 Kg. Seven subjects received intravenous infusions of a 10 per cent fructose solution with electrolytes at a rate of 2 Gm. per Kg. per hour and eight subjects received a glucose solution of similar electrolyte concentration at the same rate. The duration of the infusion was six hours except for two fructose studies which were limited to four hours because of obvious evidence of progressive acidosis.

The infused solutions were similar to the 10 per cent hexose solutions of the first experiments, except that they contained somewhat less chloride (approximately 35 mEq. per L.). The infusion technique was the same but blood sampling in these experiments was done through an indwelling Cournand needle which was placed in the femoral artery or vein 15 to 30 minutes before the start of the infusion, thus avoiding struggling during the sampling which might have elevated lactate and pyruvate concentrations in the blood. Samples were taken prior to the start, at the fourth hour, and at the end (sixth hour) of the infusion and the following studies were done: whole blood pH by a Cambridge pH meter, whole blood carbon dioxide by the technique of Van Slyke and Neill (12), plasma sodium and potassium by flame photometry, plasma chloride by the method of Schales and Schales (13), whole blood lactate by the procedure of Barker and Summerson (14), and whole blood pyruvate by the direct method of Friedemann and Haugen (15) which measures total keto acids rather than pyruvate alone.

A total urine collection was made for the period of the infusion plus the following hour and kept at a pH of approximately 1.0 in ice. The urine was analyzed for sodium, potassium, lactate, pyruvate and total reducing substance by the methods used above and for fructose by the Roe method as modified by Higashi and Peters (16).

RESULTS

Studies on utilization

The rate of infusion of the subjects in Group A (Table II) averaged 1.07 Gm. per Kg. per hour (range, 0.89 to 1.22) and 1.06 (range, 0.81 to 1.30) for glucose and fructose, respectively. In these experiments, the retention of both sugars was essentially complete and equal as indicated by the fact that only 1.6 per cent and 3.6 per cent of

TABLE II

Balance data during hexose infusions—Group A

Subject	Infusion time *hrs.*		Water *ml.*		Hexose *Gm.*		Na *mEq.*		K *mEq.*	
			Glucose	Fructose	Glucose	Fructose	Glucose	Fructose	Glucose	Fructose
Subj. 1 (Fe)	6	Intake								
age, 7 mo.		perKg./hr.	21.4	16.0	1.09	0.81	0.72	0.54	0.37	0.27
wt., 7.7 Kg.		total	990	740	50.4	37.3	33.2	24.8	17.2	12.6
		Output	826	400	0.3	0.9	22.0	2.7	12.2	10.4
		Retention	164	340	50.1	36.4	11.2	22.1	5.0	2.2
		% Excreted	83.4	54.0	0.6	2.4	66.3	10.9	70.9	82.5
Subj. 2 (Fu)	6	Intake								
age, 7 mo.		per Kg./hr.	17.5	21.0	0.89	1.06	0.58	0.70	0.30	0.36
wt., 8.8 Kg.		total	923	1,111	47.0	56.0	30.9	37.2	16.1	18.9
		Output	863	730	1.5	2.4	11.0	22.7	7.1	7.7
		Retention	60	381	45.5	53.6	19.9	14.5	9.0	11.2
		% Excreted	93.5	65.7	3.2	4.3	35.6	61.0	44.1	40.7
Subj. 3 (Re)	4	Intake								
age, 4 mo.		per Kg./hr.	23.9	25.8	1.22	1.30	0.80	0.86	0.42	0.44
wt., 7.2 Kg.		total	690	743	35.1	37.4	23.1	24.9	12.0	12.6
		Output	515	590	0.4	1.5	2.4	11.8	4.4	6.3
		Retention	175	153	34.7	35.9	20.7	13.1	7.6	6.3
		% Excreted	74.6	79.4	1.1	4.0	10.4	47.4	36.7	50.0
Average		Intake								
		per Kg./hr.	20.9	20.9	1.07	1.06	0.70	0.70	0.36	0.36
		% Excreted	83.8	66.4	1.6	3.6	37.4	39.8	50.6	57.7

the infused carbohydrates were excreted in the glucose and fructose experiments, respectively. No noteworthy differences were seen in the excretions of water, sodium and potassium in relation to the sugar infused.

The infusions administered to the eight subjects in Group B (Table III) were carried out at a rate known to exceed the infants' capacity for essentially complete utilization of glucose. Favorable circumstances were created thereby for the possible demonstration of a greater capacity for fructose utilization. The average percentage of the hexose intake excreted in the urine in the glucose experiments, 20.0 per cent, was significantly greater than that of the fructose experiments, 9.9 per cent ($p < 0.02$). The average sodium excretion associated with glucose infusion was 79 per cent of the intake, which is significantly

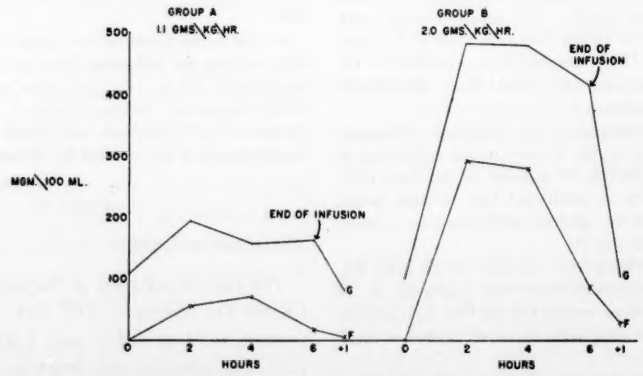

FIG. 1. AVERAGE BLOOD LEVELS DURING AND ONE HOUR AFTER INFUSION

The dots represent blood levels of total reducing substance during and after glucose infusion. The crosses are blood levels with fructose infusion.

TABLE III

Balance data during hexose infusions—Group B

Subject	Infusion time *hrs.*		Water *ml.* Glucose	Water *ml.* Fructose	Hexose *Gm.* Glucose	Hexose *Gm.* Fructose	Na *mEq.* Glucose	Na *mEq.* Fructose	K *mEq.* Glucose	K *mEq.* Fructose
Subj. 4 (Ha)	6	Intake								
age, 6 mo.		per Kg./hr.	21.8	20.1	1.57	1.44	0.72	0.67	0.37	0.33
wt., 8.5 Kg.		total	1,111	1,024	80.2	73.6	36.7	34.3	19.1	17.1
		Output	841	776	15.6	5.9	29.5	24.8	11.9	8.5
		Retention	270	248	64.6	67.7	7.2	9.5	7.2	8.6
		% Excreted	75.7	75.8	19.4	8.0	80.4	72.3	62.3	49.7
Subj. 5 (Ch)	6	Intake								
age, 3 mo.		per Kg./hr.	27.5	27.2	1.98	1.96	0.91	0.91	0.47	0.45
wt., 6.5 Kg.		total	1,072	1,063	77.4	76.4	35.4	35.6	18.4	17.7
		Output	930	929	12.1	8.6	25.6	50.2	11.2	11.7
		Retention	142	134	65.3	67.8	9.8	−14.6	7.2	6.0
		% Excreted	86.7	87.4	15.6	11.2	72.3	141.0	60.9	66.1
Subj. 6 (Gr)	6	Intake								
age, 9 mo.		per Kg./hr.	30.5	30.0	2.20	2.16	1.00	1.00	0.52	0.50
wt., 5.8 Kg.		total	1,062	1,045	76.7	75.1	35.0	35.0	18.3	17.4
		Output	892	878	20.5	10.0	31.5	42.1	9.3	8.8
		Retention	170	167	56.2	65.1	3.5	−7.1	9.0	8.6
		% Excreted	84.0	84.0	26.7	13.3	90.0	120.3	50.8	50.6
Subj. 7 (Ro)	6	Intake								
age, 5 mo.		per Kg./hr.	21.7	22.3	1.57	1.60	0.72	0.74	0.37	0.37
wt., 5.7 Kg.		total	744	762	53.7	54.8	24.5	25.5	12.8	12.7
		Output	498	672	1.9	4.1	11.5	36.1	4.9	7.6
		Retention	246	90	51.8	50.7	13.0	−10.6	7.9	5.1
		% Excreted	66.9	88.2	3.5	7.5	46.9	141.6	38.3	59.8
Subj. 8 (Ba)	6	Intake								
age, 5 mo.		per Kg./hr.	27.1	25.7	1.96	1.85	0.89	0.86	0.47	0.43
wt., 6.4 Kg.		total	1,042	989	75.2	71.1	34.4	33.1	17.9	16.5
		Output	873	825	17.4	5.5	29.4	34.1	9.6	6.8
		Retention	169	164	57.8	65.6	5.0	−1.0	8.3	9.7
		% Excreted	83.8	83.4	23.1	7.7	85.5	103.0	53.6	41.2
Subj. 9 (Ch)	6	Intake								
age, 15 mo.		per Kg./hr.	25.1	25.1	1.81	1.80	0.83	0.84	0.43	0.42
wt., 9.7 Kg.		total	1,459	1,459	105.3	104.9	48.1	48.9	25.1	24.4
		Output	1,035	1,108	23.7	5.7	29.4	55.5	14.6	20.3
		Retention	424	351	81.8	99.2	18.7	−6.6	10.5	4.1
		% Excreted	70.9	75.9	22.5	5.4	61.1	113.5	58.2	83.2
Subj. 10 (Ke)	4	Intake								
age, 2 mo.		per Kg./hr.	23.4	23.3	2.37	2.36	0.76	0.77	0.40	0.39
wt., 5.4 Kg.		total	506	504	51.3	51.0	16.4	16.6	8.6	8.4
		Output	400	333	9.3	7.8	14.1	24.9	1.9	3.7
		Retention	106	171	42.0	43.2	2.3	−8.3	6.7	4.7
		% Excreted	79.0	66.1	18.1	15.3	86.0	150.0	22.1	44.0
Subj. 11 (Li)	6	Intake								
age, 7 mo.		per Kg./hr.	22.3	25.7	2.26	2.60	0.72	0.85	0.38	0.43
wt., 6.7 Kg.		total	1,018	1,033	103.2	104.6	33.1	34.1	17.3	17.2
		Output	858	873	31.8	11.1	36.4	58.6	13.6	12.8
		Retention	160	160	71.4	93.2	−3.3	−24.5	3.7	4.4
		% Excreted	84.3	84.5	30.8	10.6	110.0	171.8	78.6	74.4
Subj. 12 (Ch)	6	Intake								
age, 16 mo.		per Kg./hr.		25.6		2.59		0.84		0.43
wt., 10.0 Kg.		total		1,534		155.4		50.6		25.6
		Output		1,101		24.2		91.4		18.8
		Retention		433		131.2		−40.8		6.8
		% Excreted		71.8		15.6		180.6		73.4
Average*		Intake								
age, 6.5 mo.		per Kg./hr.	24.9	24.9	1.96	1.97	0.82	0.83	0.43	0.41
Wt., 6.8 Kg.				p>0.9		p>0.8		p>0.5		p>0.3
		% Excreted	78.9	80.7	20.0	9.87†	79.0	126.7†	53.1	58.6
				p>0.6		p<0.02		p<0.01		p>0.3

* Averages excluding Subject 12. † Significant differences.

lower than that noted with fructose, 127 per cent ($p < 0.01$).

There were no significant differences related to the sugar infused in the intakes of water, sodium, potassium and hexose, nor in the percentages of the potassium and water intakes excreted in the urine.

The average blood levels for glucose and fructose at two hourly intervals during infusion and one hour postinfusion are illustrated in Figure 1. In agreement with the findings of other observers (1, 3) levels of total reducing substance were found to be significantly higher during glucose infusion. The concentrations tended to fall during the course of infusion of both sugars although the tendency was more marked in the case of fructose and especially so at the higher rate of infusion.

The only significant alteration in blood electrolytes was noted in the fructose infusions in Group B and consisted of a fall in serum sodium from a mean initial level of 149 to a final level of 140 mEq. per L. This finding correlates with the negative sodium balance noted in these subjects. Variations in hematocrit were without significance in either group.

No significant alterations in pulse and respiratory rates and liver size were noted during the infusions to the subjects in Group A. The average values for these measurements at the start and end of the infusions administered to the subjects in Group B are given in Table IV. Statistically significant changes were noted in all three only during the fructose infusion. The alterations observed were attributed to differences in the metabolism of fructose, because the rates of administration of water, electrolytes and carbohydrates were the same in the experiments with both sugars.

During the latter part of a fructose infusion in Subject 12, administered at the very rapid rate of 2.59 Gm. per Kg. per hour, marked hyperventilation, tachycardia, pallor and mottling appeared. Blood was drawn in an effort to determine whether this reaction was the result of circulatory or metabolic causes. The pH of venous blood was found to be 6.85 and the CO_2 content 5.5 mM per L. One hour after termination of the infusion, the levels had increased to 7.02 and 13 mM per L., respectively, coincident with marked improvement in the patient's condition. Because of the severity of the disturbance accompanying rapid fructose infusion in this subject, it did not seem justifiable to subject him to further study by administering a comparable glucose infusion. Nevertheless, the possibility suggested itself that the significant rises in pulse and respiratory rate which occurred only during fructose infusion might reflect the presence of a similar metabolic acidosis in the group of subjects infused, at a rapid rate, with this carbohydrate. Further investigation, bearing on this question, was undertaken.

Study of acid-base changes

Complete data on the blood levels found and differences existing between initial and final blood levels in each subject studied are recorded in

TABLE IV

Pulse and respiration rates and liver size at beginning and end of infusion—Subjects in Group B

	Pulse rate				Respiratory rate				Liver size*			
	Glucose		Fructose		Glucose		Fructose		Glucose		Fructose	
Subject	I†	F‡	I	F	I	F	I	F	I	F	I	F
4	150	124	90	124			32	58	3.0	2.0	2.0	2.0
5	98	106	110	160	36	36	36	44	2.0	2.0	0	2.0
6	122	132	144	159	24	32	38	38	1.0	2.0	1.0	3.0
7	140	132	140	144	36	28	50	70	1.5	1.5	2.0	3.0
8	120	120	128	128	40	30	36	64	2.0	2.0	2.0	3.0
9	124	120	146	152	40	28	32	32	2.5	3.0	0.5	4.5
10	160	200	128	160	28	32	32	48	0	1.5	1.0	3.0
11	120	160	156	160	36	40	32	42	2.0	2.5	2.0	3.5
Mean	129	137	130	148	34	32	36	49	1.7	2.1	1.3	3.0
p	>0.3		<0.05		>0.7		<0.01		>0.2		<0.01	

* Measured as cm. palpable below costal margin at midclavicular line.
† Initial.
‡ Final.

TABLE V

Blood analyses during hexose infusion

Subject	Time *hrs.*	pH	CO_2 *mEq./L.*	Na *mEq./L.*	K *mEq./L.*	Cl *mEq./L.*	Lactate *mEq./L.*	Pyruvate *mEq./L.*
				Fructose infusion				
R. Ro.	0	7.30	18.3	136	5.3	104	1.4	0.2
1	4	7.29	14.6	130	5.7	102	3.8	0.3
	Δ	−0.01	−3.7	−6	+0.4	−2	2.4	0.1
V. Lo.	0	7.33	18.8	140	4.6	105	1.5	0.2
2	4	7.12	9.1	119	5.7	104	6.7	0.5
	6	7.11	7.9	125	6.5	101	7.0	0.5
	Δ	−0.22	−10.9	−15	+1.9	−4	5.5	0.3
L. Gl.	0	7.38	19.4	141	5.0	104	1.4	0.1
3	4	7.28	14.9	131	6.5	102	3.5	0.3
	6	7.24	14.6	126	6.2	103	4.3	0.4
	Δ	−0.14	−4.8	−15	+1.2	−1	2.9	0.3
R. Or.	0	7.31	18.7	139	4.3	107	1.4	0.2
4	6	7.20	13.0	124			5.7	0.4
	Δ	−0.11	−5.7	−15			4.3	0.2
R. Ze.	0	7.41	18.2	135	4.8	101	1.6	0.2
5	4	7.29	11.6	118	4.7	98	6.4	0.5
	Δ	−0.12	−6.6	−17	−0.1	−3	4.8	0.3
D. La.	0	7.42	19.1	140	4.8	104	1.4	0.2
6	4	7.32	12.5	129	5.0	101	6.0	0.4
	6	7.28	11.5	121	4.7	99	5.4	0.5
	Δ	−0.14	−7.6	−19	−0.1	−5	4.0	0.3
R. Ma.	0	7.35	19.3	140		101	1.4	0.2
7	4	7.25	11.9	135	4.4	102	5.4	0.3
	6	7.21	10.5	129	4.8	100	5.2	0.3
	Δ	−0.14	−8.8	−11		−1	3.8	0.1
	m		−6.8	−14	+0.66	−2.7	3.96	0.24
				Glucose infusion				
D. Ga.	0	7.38	19.1	137	4.2	103	1.2	0.2
1	4	7.29	19.9	132	3.8	100	2.8	0.3
	6	7.32	22.6	133	4.6	100	3.9	0.4
	Δ	−0.06	+3.5	−4	+0.4	−3	2.7	0.2
P. Ya.	0	7.36	17.8	137	4.6	100	1.0	0.2
2	4	7.38	15.8	128	4.2	99	2.1	0.3
	6	7.38	17.0	126	4.0	98	2.2	0.3
	Δ	+0.02	−0.8	−11	−0.6	−2	1.2	+0.1
L. Hi.	0	7.39	18.5	137	5.3	100	1.2	0.2
3	4	7.38	16.5	134	4.7	98	4.1	0.4
	6	7.37	15.7	131	4.8	101	3.9	0.4
	Δ	−0.02	−2.8	−6	−0.5	+1	2.7	0.2
J. Ga.	0	7.40	19.0	138	3.8	103	1.5	0.2
4	4	7.37	18.6	129	3.8	99	2.3	0.2
	6	7.36	18.9	126	3.9	100	2.9	0.3
	Δ	−0.04	−0.1	−12	+0.1	−3	1.4	0.1
D. Da.	0	7.40	19.3	139		101	1.4	0.2
5	6	7.37	18.2	135	5.4	101	2.1	0.3
	Δ	−0.03	−1.1	−4		0	0.7	0.1
R. Pr.	0	7.40	19.3	139	3.9	102	1.4	0.1
6	4	7.37	19.1	137	3.8	102	3.8	0.3
	6	7.38	18.5	135	3.7	103	3.9	0.3
	Δ	−0.02	−0.8	−4	−0.2	+1	2.5	0.2
E. Ca.	0	7.41	19.1	137		103	1.4	0.1
7	4	7.37	18.5	135		101	2.2	0.2
	6	7.40	18.7	131	3.7	101	2.2	0.2
	Δ	−0.01	−0.4	−6		−2	0.8	0.1
	m	−0.02	−0.4	−6.7	0.16	−1.1	1.70	0.11
	p	<0.01	<0.001	<0.01	>0.1	>0.1	<0.01	<0.01

Table V. The means of the changes in each of the blood levels have been calculated separately for the group of subjects receiving fructose and those receiving glucose. The significance of the difference in these means is recorded in Table V.

The data demonstrate significantly greater rises in blood lactic and pyruvic acids in the subjects receiving fructose, than in those infused with glucose. The mean increases in lactate were 35.6 mg. per cent (3.96 mEq. per L.) and 15.3 mg. per cent (1.71 mEq. per L.) for the fructose and glucose groups, respectively. Blood pyruvate rose an average of 2.16 mg. per cent (0.24 mEq. per L.) with fructose and 0.98 mg. per cent (0.11 mEq. per L.) with glucose. Accompanying the acid metabolite accumulation noted with fructose

TABLE VI

Balance data

Subject	Infusion time *hrs.*		H_2O *ml.*	CHO *Gm.*	Na *mEq.*	K *mEq.*	Lactate *mEq.*	Pyruvate *mEq.*
			Fructose infusion					
R. Ro.	4	Intake						
Male		per Kg./hr.	22.1	2.0	0.60	0.30		
6 mos.		total	638	57.8	17.3	8.7		
7.2 Kg.		Output	322	6.8	15.0	8.3	0.96	0.17
		Retention	316	51.0	2.3	0.4		
		% Excreted	50.5	11.8	86.7	95.4		
V. Lo.	6	Intake						
Female		per Kg./hr.	20.3	2.03	0.61	0.30		
8 mos.		total	1,034	103.4	31.0	15.5		
8.5 Kg.		Output	737	9.9	42.0	8.9	6.83	0.52
		Retention	297	93.5	−11.0	6.6		
		% Excreted	71.3	9.6	135.5	57.4		
L. Gl.	6	Intake						
Female		per Kg./hr.	19.6	1.96	0.59	0.29		
2 yrs.		total	1,492	149.2	44.8	22.4		
12.7 Kg.		Output	1,022	11.6	49.2	11.3	5.72	0.99
		Retention	470	137.6	−4.4	11.1		
		% Excreted	68.5	7.8	109.8	50.4		
R. Or.	6	Intake						
Female		per Kg./hr.	20.0	2.0	0.60	0.30		
5 mos.		total	721	72.1	21.6	10.8		
6.0 Kg.		Output	508	7.4	20.5	8.8	4.38	0.56
		Retention	213	64.7	+1.1	+2.0		
		% Excreted	70.4	10.3	94.9	81.5		
R. Ze.	4.25	Intake						
Male		per Kg./hr.	19.4	1.94	0.58	0.29		
3 mos.		total	470	47.0	14.1	7.0		
5.7 Kg.		Output	302	2.6	12.5	4.3	1.69	0.25
		Retention	168	44.4	1.6	2.7		
		% Excreted	64.2	5.5	88.6	61.4		
D. La.	6	Intake						
Male		per Kg./hr.	20.4	2.04	0.61	0.31		
2.5 mos.		total	675	67.5	20.2	10.1		
5.5 Kg.		Output	486	6.7	16.1	6.2	7.43	0.63
		Retention	189	60.8	4.1	3.9		
		% Excreted	72.0	9.9	79.7	61.4		
R. Ma.	6	Intake						
Male		per Kg./hr.	20.5	2.05	0.61	0.31		
6 mos.		total	848	84.8	25.4	12.7		
6.9 Kg.		Output	527	4.4	25.3	6.4	6.20	0.44
		Retention	321	80.4	+0.1	6.3		
		% Excreted	62.1	5.2	99.6	50.4		
		Intake						
m		per Kg./hr.	20.3	2.00	0.60	0.30		
m		% Excreted	65.6	8.6	99.2	65.4		

TABLE VI—*Continued*

Subject	Infusion time *hrs.*		H_2O *ml.*	CHO *Gm.*	Na *mEq.*	K *mEq.*	Lactate *mEq.*	Pyruvate *mEq.*
			Glucose infusion					
D. Ga.	6	Intake						
Female		per Kg./hr.	21.6	1.94	0.62	0.30		
11 mos.		total	1,049	94.3	30.0	14.7		
8.1 Kg.		Output	691	10.2	25.6	6.6	0.32	0.24
		Retention	358	84.1	4.4	8.1		
		% Excreted	65.9	11.9	85.3	44.9		
P. Ya.	6	Intake						
Female		per Kg./hr.	19.8	1.98	0.61	0.31		
18 mos.		total	1,083	108.3	33.6	16.9		
9.1 Kg.		Output	748	44.2	24.7	17.7	0.21	0.10
		Retention	335	64.1	8.9	−0.8		
		% Excreted	69.1	40.8	73.5	104.7		
L. Hi.	6	Intake						
Male		per Kg./hr.	20.2	2.10	0.62	0.30		
2.5 mos.		total	643	66.7	19.6	9.6		
5.3 Kg.		Output	491	12.8	20.9	4.4	0.54	0.15
		Retention	162	53.4	−1.3	5.2		
		% Excreted	74.8	19.2	106.6	45.8		
J. Ga.	6	Intake						
Male		per Kg./hr.	19.9	1.78		0.31		
7 mos.		total	1,094	98.0		17.1		
9.15 Kg.		Output	753	28.6		6.0	0.49	0.29
		Retention	341	69.4		11.1		
		% Excreted	68.8	29.2		35.1		
D. Da.	6	Intake						
Male		per Kg./hr.	19.6	1.80	0.61	0.30		
8 mos.		total	823	75.6	25.7	12.7		
7.0 Kg.		Output	487	14.0	12.4	4.7	0.16	0.15
		Retention	336	61.6	13.3	8.0		
		% Excreted	59.4	18.5	48.2	37.0		
R. Py.	6	Intake						
Male		per Kg./hr.	19.9	1.93	0.62	0.30		
14 mos.		total	1,362	132.6	42.2	20.8		
11.4 Kg.		Output	962	21.2	34.6	6.5	0.54	0.21
		Retention	400	111.6	7.6	14.3		
		% Excreted	70.6	16.0	82.0	31.2		
E. Ca.	6	Intake						
Male		per Kg./hr.	20.1	1.99	0.62	0.31		
8 mos.		total	1,038	102.8	32.2	16.2		
8.6 Kg.		Output	801	30.8	29.6	11.9	0.15	0.12
		Retention	237	72.0	2.6	4.3		
		% Excreted	77.2	30.0	91.9	73.4		
		Intake						
m		per Kg./hr.	20.2	1.94	0.62	0.30		
m		% Excreted	69.4	23.6	81.2	53.1		
p			>0.3	<0.01	>0.1	>0.3	<0.001	<0.01

infusion, decreases in blood pH ranging from 0.11 to 0.22 were seen in six of the seven subjects, and mean decreases in whole blood CO_2 of 6.8 mM per L. (range, 3.7 to 10.0 mM per L.), and in plasma sodium of 14 mEq. per L. (range, 6 to 19 mEq. per L.) were found. These values likewise differ significantly from those noted in the glucose group, where decreases in blood pH ranged from 0.01 to 0.06 and mean decreases in CO_2 of 0.4 mM per L. (range, − 2.8 to + 3.5 mM per L.), and in sodium of 6.7 mEq. per L. (range, 4 to 12) were seen. No significant differences in potassium or chloride levels were demonstrated.

Table VI presents data for intake and excretion of water, carbohydrate, sodium and potassium, as well as excretion of lactate and pyruvate of the

individual subjects. As in the previous study, the excretion of fructose (8.6 per cent of intake) was significantly lower than that of glucose (23.6 per cent), but in contrast to the earlier group of subjects, the children who received fructose in general did not show a negative sodium balance. The mean sodium excretion of the individuals receiving fructose was higher (99.2 per cent of intake) than that noted with the glucose infusions (81.2 per cent), but not significantly so. The greater mean sodium loss noted in the first study probably reflects the very high infusion rates used in several of the subjects. As before, no significant difference was seen in water or potassium excretions with the two carbohydrates.

A highly significant difference was found in the lactic acid excretions of the two groups, 10.4 mg. per Kg. per hour and 0.6 mg. per Kg. per hour for the subjects receiving fructose and glucose, respectively. A significant but less striking difference existed in pyruvate excretion, which averaged 0.6 mg. per Kg. per hour with fructose administration and 0.3 mg. per Kg. per hour with glucose.

DISCUSSION

The results reported here established the fact that infants can utilize fructose almost completely when it is administered at the relatively rapid rate of 1.0 Gm. per Kg. per hour for periods of at least six hours. This rate is equivalent to the maximum set for glucose by Butler and Talbot (7) and confirmed for infants in the present study. When the two sugars were administered at an average rate of 2.0 Gm. per Kg. per hour in order to demonstrate a possible superiority in fructose utilization, there was a statistically significant difference in utilization in favor of fructose.

As stated above, there were indications that the superior utilization of fructose, at rapid rates of infusion, was not entirely advantageous. Statistically significant increments in pulse and respiratory rate were demonstrated during fructose infusion, and appeared to be manifestations of cardiorespiratory responses to an altered metabolic state. A significant enlargement of the liver was likewise noted in association with rapid fructose infusion, probably as the result of the more rapid uptake (17, 18) and conversion of this sugar to glycogen (19). In addition, the subjects in Group B exhibited an average negative sodium balance with fructose infusion but not with glucose. This difference was not noted in the subsequent series of experiments in which the carbohydrate infusion rate was maintained more uniformly around 2.0 Gm. per Kg. per hour. Negative sodium balances were particularly prominent in the individual experiments in Group B in which the infusion rate varied above the mean for the group as a whole.

These indications of the existence of a metabolic acidosis were borne out by further investigation of blood pH, CO_2 and electrolyte changes. The probable mechanism for this acidosis was demonstrated to be an accumulation in the blood of lactic and pyruvic acids with increased urinary excretion of these metabolites. Similar elevation in blood levels of these intermediates has been reported previously from several sources (8, 17, 18).

The present controlled studies show that the magnitude of these metabolic alterations is significantly greater with fructose administration than with glucose. The increased appearance of these metabolites occurring especially with fructose administration is probably a reflection of its rapid rate of utilization which has been attributed to a difference in rate of phosphorylation (17) and to the fact that fructose enters the Meyerhof pathway at a point further along than glucose (17). Smith, Ettinger, and Seligson (18) have suggested that the difference in acid metabolite accumulations is a reflection of the significant extent to which glucose oxidation in the human proceeds through the hexosemonophosphate shunt pathway, in which only 1 M of pyruvate is formed from the breakdown of 1 M of hexose, while 2 M of pyruvate is produced in the Embden-Meyerhof pathway, which is the only known mechanism for fructose breakdown.

The subjects used in these studies had no preexisting acidosis, but showed blood pH decreases to as low as 7.11 during the course of a six hour infusion. Even more profound acidosis could be expected to occur in a patient who was acidotic before the infusion was begun. This situation might be encountered in a diabetic, acutely undernourished, or dehydrated infant.

The acidosis accompanying rapid fructose administration in infants has been shown to be of sufficient severity to make advisable limitation of its rate of infusion. No untoward side effects were

noted with infusions of 1 Gm. per Kg. per hour and no contraindication to its use at this rate has been demonstrated.

It should be pointed out that there may be indications for the use of fructose in special situations in which glucose utilization may be impaired: the initial hours of treatment of diabetic acidosis (20, 21), febrile illness (22), the early postoperative period (22), severe hepatic insufficiency (18), after fasting (23), and in the acidotic state (24). The present study does not yield information relevant to the validity of these indications.

SUMMARY

1. Infusions of glucose and fructose with electrolytes to three infants at a rate of approximately 1.0 Gm. per Kg. per hour resulted in essentially complete utilization of both sugars. Excretion of water, sodium, and potassium did not vary significantly in relation to the sugar infused.

2. Infusions of the sugars at a rate of approximately 2.0 Gm. per Kg. per hour in eight subjects resulted in a significantly greater urinary loss of sugar with glucose (20.0 per cent of intake) than with fructose (9.9 per cent) ($p < 0.02$).

The average urinary sodium excretion associated with glucose infusion was 79 per cent of intake, which is significantly lower than that noted with fructose, 127 per cent ($p < 0.01$), but no significant differences in water and potassium excretions were noted.

Significant increases in pulse and respiratory rates and liver size occurred during fructose infusion in these subjects.

3. Infusions of fructose solution with electrolytes to seven additional infants at the rate of 2 Gm. per Kg. per hour were accompanied by mean increases in whole blood lactate of 35.6 mg. per cent, and in pyruvate of 2.16 mg. per cent. Smaller increases occurred with glucose in eight subjects.

Decreases in blood pH up to 0.22, and mean decreases in whole blood CO_2 of 6.8 mM per L. and in plasma sodium of 14 mEq. per L. were demonstrated in the group of individuals receiving fructose. Significantly smaller changes were seen in these blood levels during glucose infusion.

Mean lactic acid excretion during the period of infusion was 10.4 mg. per Kg. per hour in the subjects receiving fructose, but only 0.6 mg. per Kg. per hour in those receiving glucose.

4. It is suggested that the advantage in fructose utilization over glucose, when administered at a rapid rate of infusion, may be offset by the induced metabolic acidosis and its attendant side effects.

ADDENDUM

Since preparation of this manuscript, another study of the use of carbohydrates in parenteral feeding of children has been reported by Lane and Dodd (25). They administered 10 per cent solutions of glucose, invert sugar, and fructose, containing no electrolytes, to 18 children and found more glycosuria accompanying the glucose infusions than with the fructose or invert sugar in 16 of the subjects. Their rate of infusion was 1.25 Gm. of sugar per Kg. per hour, which is considerably lower than the 2 Gm. per Kg. per hour rate which produced the significantly greater retention of fructose than glucose in the present study.

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PLETHSYMOGRAPHIC STUDIES OF THE CEREBRAL CIRCULATION: EVIDENCE FOR CRANIAL NERVE VASOMOTOR ACTIVITY[1, 2]

By THOMAS J. BRIDGES, KEMP CLARK,[3] AND MELVIN D. YAHR

(From the Departments of Neurological Surgery and Neurology, College of Physicians and Surgeons, Columbia University, The Neurological Institute, Presbyterian Hospital, and the Francis Delafield Hospital, New York, N. Y.)

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Cerebral vasodilatation and vasoconstriction may be recorded by the electronically-amplifying pneumoplethysmograph. This method represents an adaptation of the plethysmographic method of recording digital vasomotor changes. The brain is enclosed in a largely nondistensible dural envelope (1), which, in effect, is a naturally-occurring plethysmograph chamber. The dura, backed by the skull, is a substitute for the rigid plastic cup used for digital plethysmography (2).

Cerebral plethysmographic responses to a number of vasomotor stimuli and to cervical sympathetic nerve stimulation and nerve block are reported.

Early efforts (1881 to 1929) to relate change in cerebral circulation to differences in amplitude of pulsation over human cranial defects, as recorded by the kymograph, are summarized by Shepard (3) and Stevenson, Christensen, and Wortis (4). These observations could be obtained from subjects with large cranial defects capable of transmitting a volume-pressure change sufficient to activate a mechanical recording system. Electronic amplification permits volume pulse recordings to be obtained through a small cranial trephination.

Cerebral vasodilator and vasoconstrictor agents have been identified by a method of pial vessel photography and measurement (5, 6), and by calculation from dye-dilution (7) and nitrous oxide (8) blood flow methods. Vasomotor stimuli of predictable action may be utilized in the evaluation of a cerebral plethysmographic method.

METHOD AND MATERIAL

One of two electronically-amplifying, direct recording plethysmographs is connected to the human subdural space in an air-and-water-tight fashion. A small trephination is made in the temporal region of the skull under local anesthesia. The dura is opened widely. Effort is made to preserve the integrity of the arachnoid. A chambered, cast vitallium, trephine button is inserted into the trephination opening in the skull. A short length of plastic tubing is threaded onto the stem of the trephine button and led out through a separate stab wound in the scalp. The button is then connected to the recording plethysmograph by means of 10 to 15 feet of plastic tubing. Recordings may be obtained for periods up to 23 days. After this time, the dura will be reformed, and pulsations will be considerably damped. At the conclusion of the recording period, the plastic tubing is gently pulled away from the cast vitallium trephine button and removed from the stab wound, allowing this wound to heal. The trephine button may be left *in situ* indefinitely, avoiding the need to perform further surgery to remove the button.

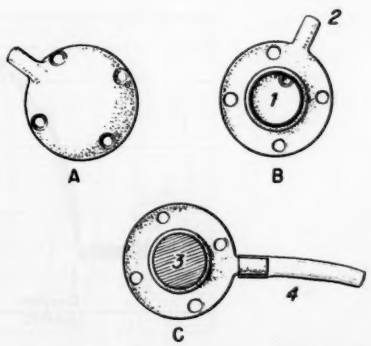

FIG. 1. DIAGRAM OF CAST VITALLIUM TREPHINE BUTTON

A. External surface of volume recording button.

B. and C. Internal surface of recording button.

1) Volume transmitting air chamber; 2) volume transmitting stem; 3) lax rubber diaphragm separating cerebrospinal fluid and air; 4) Portex tubing for connection to plethysmograph.

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[2] A preliminary report of this project was presented before the Harvey Cushing Society, May 18, 1955.

[3] Present address: Southwestern Medical School, University of Texas, Dallas, Texas.

MATERIAL

The cerebral plethysmographic observations to be reported were obtained from 18 patients, 10 women and 8 men, undergoing "injection-type" lobotomy, cervicothoracic sympathectomy, or "head-and-neck dissection" operations. The age range was 26 to 48 years, with an average age for the group of 33.3 years. None of these patients had cerebrovascular, cardiovascular, or central nervous system disease. Observations of cerebral plethysmographic changes have been made in a few patients with cerebrovascular disease or brain tumor, but these observations have been purposely excluded, and will be reported when a sufficiently large number of observations will have been made.

The trephine button (*Figure 1*). The cast vitallium trephine button was designed to fit snugly into a 14 mm. trephination. The chamber of the button is open to a side tube (stem) to which No. 6 hard-walled Portex tubing attaches firmly. The inner aspect of the chamber may be left open over the arachnoid, or may be covered with a lax, thin rubber membrane to prevent infection of the subarachnoic space, or a leak of cerebrospinal fluid into the plastic tubing.

The plethysmograph. The two plethysmographs used for this study were designed and constructed to reflect changes in digital volume (9, 10). The rubber membrane of the tambour rises or falls slightly with change of volume of a digit enclosed in a rigid plastic cup or of the intradural contents. Movements of the tambour in turn stretch or relax slightly a strain gauge wire, thereby changing electrical resistance. The strain gauge forms part of a sensitive alternating current wheatstone bridge. Changes in bridge resistance are amplified and transmitted to a phase detector whose output actuates the pen of the ink recorder to produce traces.

Plethysmograms. Traces are made by the ink-writer on moving chart paper graduated with vertical curved lines, 1.0 cm. apart, and horizontal straight lines 1.0 mm. apart. Chart speed is 1.0 cm. per 10 seconds. Calibration marks (cal) indicate direction of increasing volume. The amplitude of the pulse volume chart excursion may be increased or decreased by electronic amplification (gain). A base-line pulse amplitude of 2 or 3 mm. is convenient for most observations. An increase or decrease of 0.5 mm. of pulse amplitude may be significant, but larger amplitude changes will be more easily de-

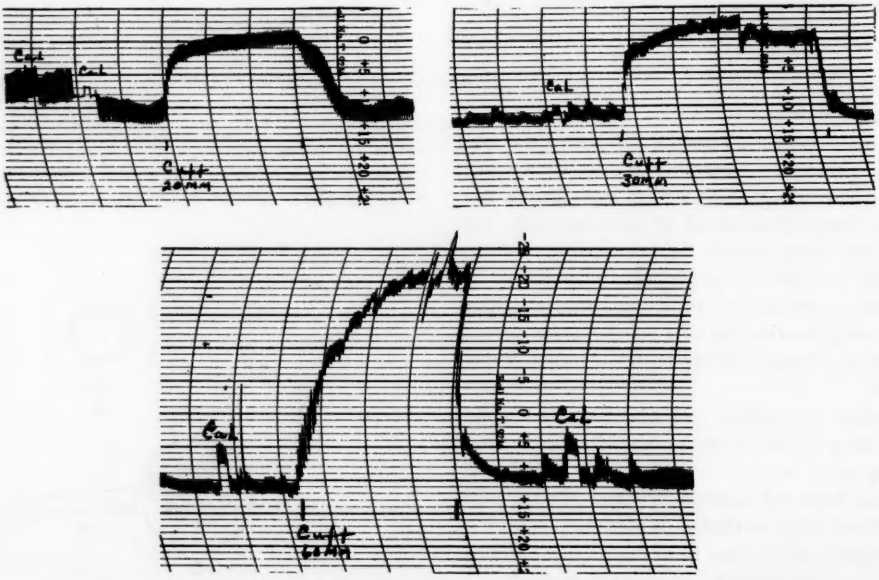

FIG. 2. EFFECT OF JUGULAR VEIN OCCLUSION ON INTRADURAL VOLUME AND PRESSURE

Jugular vein occlusion causes an increase of intradural volume and pressure, but has little effect upon pulse volume amplitude.

Manometric cuff pressure about neck	Spinal fluid pressure I.P.	Max. P.
20 mm.	90	160
30 mm.	100	180
60 mm.	100	260

tected. No quantitative significance can be attached to these arbitrary chart distance readings or changes.

Three types of volume displacement may be noted in cerebral plethysmogram traces:

1) Arterial pulse volume. Though many factors may influence pulse volume amplitude, the pulse volume excursion may be considered to be the resultant of intravascular pressure and volume forces tending to distend the pulsatile vascular field during systole as opposed by resistance of the walls of pulsating vessels to stretch. Vascular resistance to stretch will depend upon the anatomy of the vessel wall, its luminal size, and the ability of the vessels to constrict or relax. Change of pulse volume amplitude is the most sensitive plethysmographic response to cerebral vasomotor change.

2) Total, or intradural, volume deflection may represent change of brain, blood, or cerebrospinal fluid volume. Fortunately, intradural volume (and also arterial pulse amplitude) may remain constant over several hours of observation. Intradural volume is changed most rapidly and frequently by a change of volume of blood within or about the dural sac. Vasoconstriction is usually, though not always, accompanied by reduced total volume, and vasodilatation may cause an increase of intradural volume. The effect of increased or decreased cerebrospinal venous pressure is frequently seen, as the valveless veins of the cerebrospinal axis readily transmit pressure changes from the chest or abdomen in a retrograde fashion, or become engorged when the patient is placed in a head-down position. When position of the patient is little changed, intradural volume remains quite constant; hence, if changes of cerebrospinal fluid volume are occurring with any frequency, they are compensated by other volume changes, probably by the degree of venous engorgement.

3) Respiratory waves may or may not appear. Propagated along the cerebrospinal veins, their amplitude is affected by venous engorgement and by respiratory effort, in order of influence. By selection of patient position for optimum venous engorgement, these waves may be caused to disappear. The arterial pulse volume is little affected by cerebrospinal pressure changes or by degree of venous engorgement, within normal limits of cerebrospinal fluid pressure (Figure 2). Greater than normal levels of cerebrospinal fluid pressure might be expected to have an effect upon pulse volume amplitude.

Damping. Initially, "undamped" recordings of changes in intracranial volume were obtained in the operating room. To permit long-term observations, the vitallium button was fitted with a thin rubber diaphragm to minimize the chance of intracranial infection and to prevent escape of cerebrospinal fluid into the conducting tubing to the plethysmograph.

In practice, the damping effect of the rubber diaphragm has been of unexpected value. Pulse volume changes of the pulsating human brain are of much greater magnitude than are those originating from the invisibly pulsating digit. Without "damping" the amplitude of cerebral

TABLE I

Cerebral vasomotor, pulse and blood pressure responses to various stimuli

	No. patients	No. obs.	Pulse rate From	Pulse rate To	Blood pressure From	Blood pressure To	Intradural vol. change mm. +	Intradural vol. change mm. −	Pulse vol. change mm. C	Pulse vol. change mm. E
Cerebral vasodilatation										
CO_2 5%–O_2 95% inhalation	6	36	82	96	120/80	135/95	10		2	25
Voluntary apnea	10	14	72	84	120/80	125/85	2		1	10
Sleep	4	20	Variable				5–7		2	5
Alcohol, 250 ml. of 10% sol., I.V.	2	3	88	84	124/78	124/78	2		2	3
Priscoline®, 50 mg., I.V.	2	2					0	0	2	3
Papaverine, 100 mg., I.V.	2	2					5		1.5	2
Facial nerve activity	2	12	84	84	115/80	115/80	5		2	4
Sup. cerv. gang. block	2	4	82	90	115/75	115/75	4		2	3
Cerebral vasoconstriction										
Hyperventilation	10	14	94	94	115/80	125/85		5	3	2
Carotid occlusion	3	14	82	82	118/80	118/80		5	3	2
Cold constrictor test	3	10	76	86	120/80	135/85	3		3	1
Abdominal pain	2	8	84	88	110/70	115/80		3	2.5	2
Cutaneous pain	4	10	76	86	120/80	135/85		5	3	2
Apprehension	3	6					4		3	1
Anesthesia	4	5	Variable		120/80	120/80			5	2
Stim. of sup. cerv. gang.	4	15	84	100	120/80	130/85		5	2.5	1
Stim. of mid. cerv. gang.	4	15	84	100	120/80	130/85		5	3	2.5
Block of stellate gang.	6	8	76	84	115/70	115/70	0	0	2.5	2
No cerebral vasomotor change										
Nicotinic acid, 200 mg., I.V.	4	6					0	0	2	2
Mental test	1	5	72	80	115/75	120/75	0	0	2	2
Stim. of stellate gang.	4	15	84	100	120/85	130/85	0	0	3	3

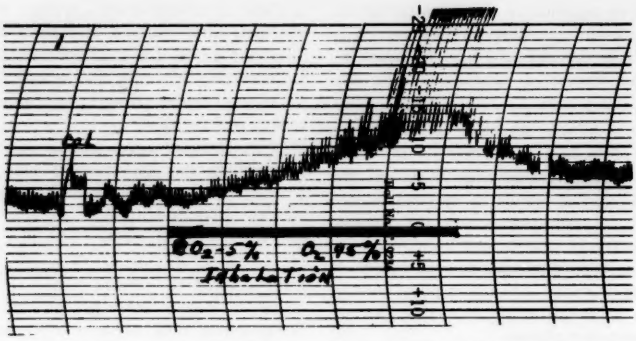

FIG. 3. EFFECT OF CARBON DIOXIDE INHALATION ON PLETHYSMOGRAPH RESPONSE

Pronounced cerebral vasodilator plethysmographic response occurs during inhalation of carbon dioxide 5 per cent-oxygen 95 per cent. There is a precipitous onset and cessation of pulse volume change during and at the termination of inhalation of the gas mixture.

pulse volume, an excursion on the chart of 3 to 6 cm. is obtained. Such a large amplitude is inconvenient for study of pulse volume change and precludes an increase of trace amplitude without being off the chart. A much less sensitive plethysmograph might be substituted for the ones used in these studies. However, it would seem preferable to use a highly sensitive plethysmograph with appropriate damping to permit detection of onset of volume change.

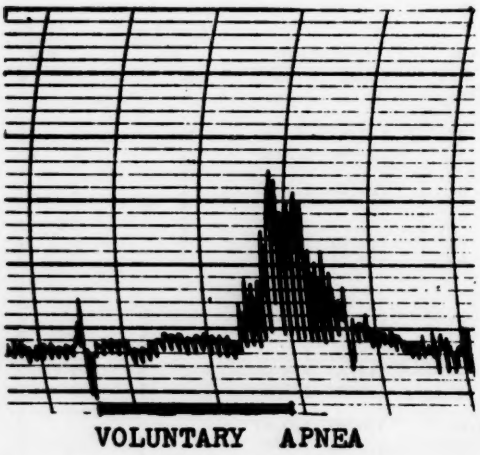

FIG. 4. EFFECT OF VOLUNTARY APNEA

Voluntary apnea (breath holding) for 22 seconds causes cerebral vasodilatation. There are no respiratory pulsations in this tracing (compare with Figure 2). Again sudden onset and cessation of vasodilatation is pronounced.

RESULTS

Cerebral vasomotor changes which occur in response to various stimuli or changing physiological states are summarized in Table I.

The number of patients studied and the number of observations (traces) for each test situation are listed. Brachial artery blood pressure, as obtained by ausculation, and pulse rate changes are recorded, and are the average of measurements taken during the total number of observations. The lowest pulse rate in any patient at any time was 64; the highest, 110. Blood pressure was within the range 90/60 to 140/100 mm. of mercury, including all test situations. Resting blood pressure was not greater than 120/80 in any patient.

The average deflection of the traces, indicating an increase or decrease of intradural (total) volume is tabulated as excursion, above (positive), or below (negative) the base line, in millimeters of chart distance.

An increase or decrease of amplitude of pulse volume is shown in the last column of Table I by comparison of the amplitude obtained during the experiment with the preceding and following control periods.

The degree of plethysmographic response varied from patient to patient and from test to test in the same patient, but the direction or type of response was consistent in this group of relatively young adults for the stimuli listed in Table I. Elderly persons, or the use of stimuli such as

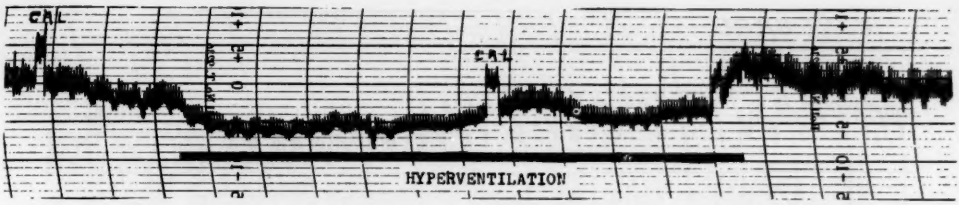

FIG. 5. EFFECT OF HYPERVENTILATION ON CEREBRAL PLETHYSMOGRAPHIC RESPONSE

Cerebral vasoconstriction occurs during hyperventilation, as indicated by reduced cerebral pulse and intradural volumes. Onset and termination of vasoconstriction are abrupt.

caffeine sodium benzoate, may show variability of response with respect to direction as well as degree.

Cerebral vasodilatation accompanies carbon dioxide 5 per cent-oxygen 95 per cent inhalation (Figure 3), voluntary apnea (Figure 4), sleep, sedative effect of intravenous alcohol, and intravenous administration of undiluted tolazoline hydrochloride (Priscoline®), 50 mg., and papaverine, 100 mg.

Cerebral vasoconstriction occurs with active hyperventilation (Figure 5), carotid occlusion, cold immersion or constrictor test, exacerbations of abdominal pain due to cancer, cutaneous painful stimulation, apprehension, and anesthesia.

Carotid artery occlusion causes reduced cerebral pulse volume and intradural volume as a "passive" vasoconstriction response to reduced blood flow and pressure.

Apprehension (fright) was produced by threatening the patient with immersion of the leg in ice water. The cold pressor test itself was carried out with reassurance and verbal support to gain maximum patient cooperation.

No cerebral vasomotor response was detected after intravenous administration of nicotinic acid, 0.2 Gm., or during performance of arithmetical or reading tests.

The cold pressor test causes reduced cerebral pulse volume, increase of pulse rate, and increased blood pressure. This pattern is consistent with cerebral vasoconstriction. Total, or intradural, volume usually increases, which might be considered vasodilatation. However, total volume increase in this instance is associated with rise of cerebrospinal venous and fluid pressure due to increased intra-abdominal and possibly intrathoracic pressure caused by obvious anxiety and muscular tension during the cold constrictor test.

Cerebral vasomotor activity following nerve stimulation. Cerebral vasodilatation accompanies facial nerve activity in the form of repetitive movement of the facial muscles (Figure 6). The patient carries out a series of "grimacing" movements, consisting of opening and closing the eyes, accompanied by alternately smiling and puckering lip movements. This obviates any apprehension which might be associated with electrical stimulation of the facial nerve. The dilator response is prompt and pronounced. Repetitive movements of the jaw muscles (fifth nerve), pharynx (ninth

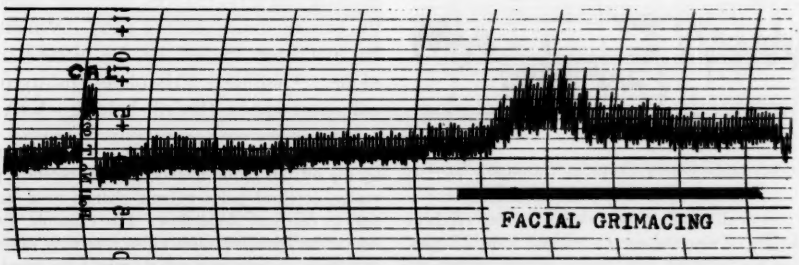

FIG. 6. CEREBRAL VASODILATATION IN RESPONSE TO VOLUNTARY REPETITIVE MOVEMENTS OF FACIAL MUSCLES

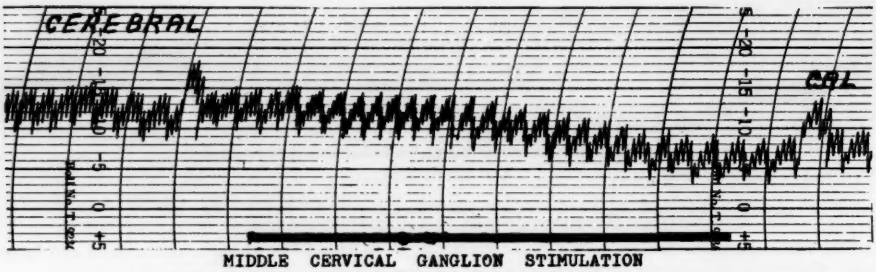

FIG. 7. EFFECT OF MIDDLE CERVICAL GANGLION STIMULATION ON CERVICAL PLETHYSMOGRAPHIC RESPONSE

Middle cervical ganglion stimulation results in cerebral vasoconstriction. Note that pulse volume decreases less strikingly than in Figure 8, but that intradural volume is decreased, and amplitude of respiratory wave increases, as with superior cervical ganglion stimulation.

and tenth nerves), or tongue (twelfth nerve) produce no change in the cerebral plethysmogram.

Cerebral vasoconstriction follows electrical stimulation of the middle or superior cervical sympathetic ganglion in anesthetized man (Figures 7 and 8). The response is more easily elicited from the superior cervical ganglion. Electrical stimulation of the stellate ganglion fails to alter the cerebral plethysmogram (Figure 9), though digital vasoconstriction is easily recorded.

Cerebral vasodilatation, indicated by increased cerebral pulse amplitude and intradural volume, accompanies procaine block of the superior cervical ganglion in the conscious subject (Figure 10). Stellate ganglion block causes a slight reduction in cerebral pulse volume without change of intradural volume (Figure 11). This is "passive vasoconstriction," as blood is shunted from the brain to the vasodilated arm. Elevation of the arm or placing the patient in a head-downward position partially restores the cerebral pulse volume toward the prestellate block amplitude.

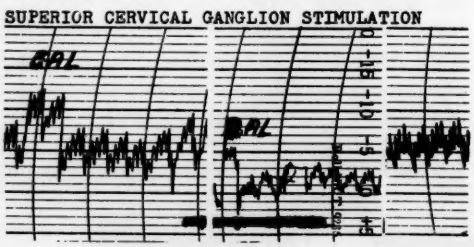

FIG. 8. EFFECT OF SUPERIOR CERVICAL GANGLION STIMULATION ON CEREBRAL PLETHYSMOGRAPHIC RESPONSE

Superior cervical ganglion stimulation causes cerebral vasoconstriction. Pulse volume is reduced from 2.5 mm. to 1 mm. One hundred and thirty second section of stimulation phase and 60 second section of recovery phase deleted to facilitate reproduction.

DISCUSSION

The plethysmographic method of recording regional vasomotor change has been adapted for the study of cerebral circulation. The plethysmographic responses may be compared with cerebral vasomotor changes which have been computed by investigators using the nitrous oxide technique for the determination of cerebral blood flow.[4] Cerebral vasodilatation has been indicated by both the cerebral plethysmographic technique and the nitrous oxide method to accompany inhalation of carbon dioxide 5 per cent-oxygen 95 per cent (11), sleep (12, 13), and administration of alcohol (14) or papavarine (15). Intravenous Priscoline® causes vasodilatation by plethysmography, but results in slightly reduced cerebral blood flow by the nitrous oxide method (16). Voluntary apnea has not been studied by the nitrous oxide technique. Undiluted Priscoline® intravenously and voluntary apnea (breath holding) have short pe-

[4] Though cerebral vasomotor activity may be indicated by the nitrous oxide method and by cerebral plethysmography, cerebral pulse volume change need have no relation to cerebral blood flow. For example, stellate ganglion stimulation increases pulse rate with unchanged pulse amplitude, a response which might be construed as indicating increased cerebral minute blood flow in the absence of cerebral vasomotor change.

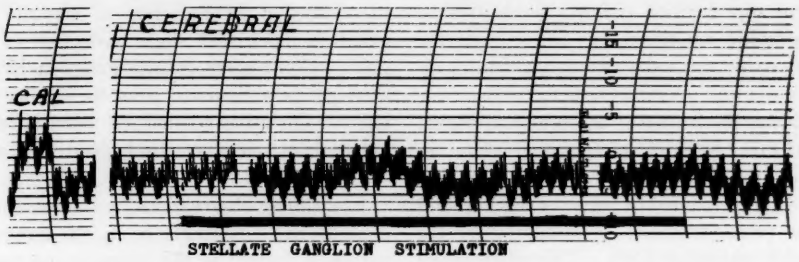

FIG. 9. EFFECT OF STELLATE GANGLION STIMULATION ON CEREBRAL PLETHYSMOGRAPHIC RESPONSE

Stellate ganglion stimulation does not change pulse or intradural volume, though increase in pulse rate may be noted.

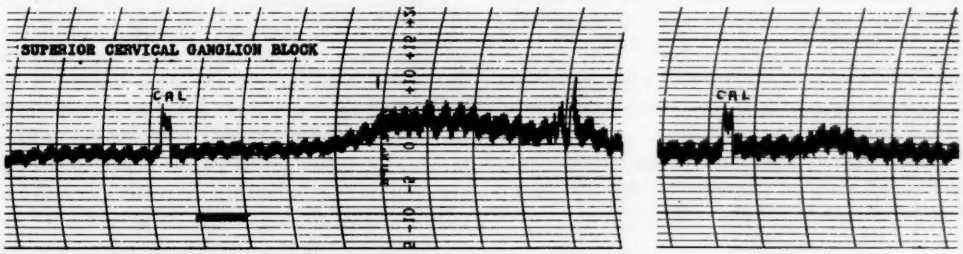

FIG. 10. PROCAINE BLOCK OF SUPERIOR CERVICAL GANGLION CAUSES CEREBRAL VASODILATATION

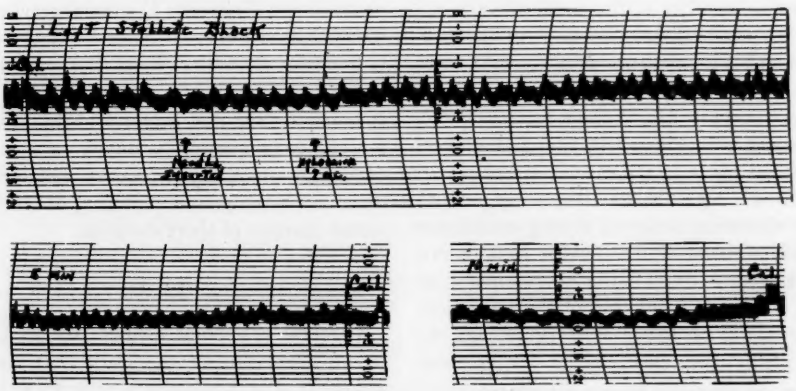

FIG. 11. EFFECT OF STELLATE GANGLION BLOCK ON CEREBRAL PLETHYSMOGRAPHIC RESPONSE

Stellate ganglion block consistently results in the reduced cerebral pulse volume of "passive" cerebral vasoconstriction, secondary to shunting of blood into vasodilated arm and face.

riods of activity, which would render their reaction more appropriate for plethysmographic study than for evaluation by the nitrous oxide method which required 5 or 10 minute periods of equilibration.

Cerebral vasoconstriction is indicated by plethysmographic and nitrous oxide methods to accompany active hyperventilation (17) and carotid artery occlusion (18). The short, though pronounced, constrictor response to the cold pressor

test, pain, or apprehension (fright), lends itself to plethysmographic recording, but is too brief for the nitrous oxide method to be conveniently applied.

Surgical anesthesia causes cerebral and digital vasoconstriction type of plethysmographic responses, whereas thiopental sodium (Pentothal®) anesthesia is accompanied by an increased cerebral blood flow (vasodilatation) as measured by the nitrous oxide technique (19). This discrepancy may be due to the complicated anesthesia mixture [thiopental sodium, nitrous oxide, oxygen, meperidine (Demerol®), and carbon dioxide absorption] administered to our subjects. Natural sleep and barbiturate- or alcohol-induced sleep are accompanied by cerebral vasodilation. Whatever the explanation may be, cerebral pulse volume amplitude is reduced by the anesthesia prior to cervical sympathetic nerve stimulation; hence further pulse volume reduction is more striking.

Mental activity and intravenous nicotinic acid have no effect upon the cerebral plethysmogram. Neither stimulus has been reported to alter cerebral blood flow (15, 20).

The neurogenic mechanisms which may influence cerebral circulation have been of particular interest to us. Activity of the facial nerve is associated with cerebral vasodilatation in the conscious human. The presence of a cerebral vasodilator mechanism involving the facial nerve was reported by Chorobski and Penfield from S. Cobb's laboratory, and the pathway of vasodilator fibers from the facial nerve to the carotid artery plexus was described (21). In the laboratory animal, pial vessel dilatation occurred during stimulation of the distal segment of the divided facial nerve. These investigators could detect no vasomotor response to electrical stimulation of the trigeminal, glossopharyngeal, or hypoglossal nerve, and we found no plethysmographic change during chewing, talking, or repetitive tongue movements.

Stimulation of the superior and middle cervical ganglia cause cerebral vasoconstriction and stellate ganglion stimulation is not accompanied by cerebral vasomotor activity in the anesthetized human. This result is similar to the observations of the effect of sympathetic stimulation upon pial vessel diameter in cat, dog and monkey, reported by Forbes, Wolff, and Cobb (5, 22–24). Cerebral vasodilatation in response to superior cervical ganglion block and slight reduction of cerebral pulse volume after stellate ganglion block occur as might be anticipated from the results of stimulation. Quantitative methods show no change or slight reduction of cerebral blood flow after stellate ganglion block (25).

From these observations, it is apparent that the concept that the outflow of all vasoconstrictor nerves is limited to the thoracic and lumbar anterior roots deserves reevaluation. Cerebral vasoconstrictor fibers are present in the cervical sympathetic nerves of animal and man, but do not traverse or involve the stellate ganglion.

Cerebral vasoconstrictor fibers pass to the superior cervical ganglia and adjacent cervical sympathetic chain from the vagus, as is found by stimulation of a segment of vagus nerve isolated at the base of the skull and in the lower part of the neck by double, procaine (4 per cent) block. Other possible routes of preganglionic fibers to the superior cervical ganglion are the ninth, eleventh and twelfth cranial and upper cervical nerves (26) and we are attempting to determine the presence or absence of vasoconstrictor fibers in these nerves. The sympathetic, vasoconstrictor system is composed of two divisions: *1*) cranial-cervical, and *2*) thoracolumbar.

Cerebral plethysmograms were obtained with surprising consistency and with little difficulty. It is our feeling that cerebral plethysmography is a useful technique for "scanning" a wide range of conditions which might produce a cerebrovascular reaction, and to permit recording of cerebral vasomotor changes of short duration.

SUMMARY

Cerebral vasomotor change has been recorded by an adaptation of the plethysmographic technique.

Increase in amplitude of cerebral pulse volume, accompanied by an increase of intradural volume, occurs in response to inhalation of a mixture of carbon dioxide 5 per cent-oxygen 95 per cent, voluntary apnea, sleep, voluntary facial nerve activity, superior cervical ganglion block, and the intravenous administration of alcohol and papaverine. We consider this to be cerebral vasodilatation.

Decrease of pulse volume amplitude with reduced intradural volume (cerebral vasoconstriction) is the plethysmographic response to hyperventilation, abdominal and cutaneous pain, and stimulation of the middle or superior cervical ganglion. The cold pressor test and apprehension cause reduced pulse amplitude and increased total volume. This is interpreted as cerebral vasoconstriction in the presence of increased cerebrospinal venous pressure due to muscular tension.

Administration of nicotinic acid, performance of mental tests, and electrical stimulation of the stellate ganglion do not affect cerebral vasomotor activity.

A craniocervical sympatheic system whose fibers appear to accompany the vagus nerve to the superior cervical ganglion has been shown to subserve cerebrovascular activity. This system is separate from the thoracolumbar sympathetic outflow.

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The authors wish to express their deep appreciation for the helpful and critical suggestions of Professor Harry Grundfest, designer of the plethysmograph used for this study.

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AN INVESTIGATION OF THE PULMONARY FUNCTION OF PARAPLEGICS [1]

By ALLAN HEMINGWAY, ERNEST BORS, AND RICHARD P. HOBBY

(From the Long Beach Veterans Administration Hospital, Long Beach, and the University of California Medical School, Los Angeles, Calif.)

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In evaluation of pulmonary insufficiency in patients with paralytic respiratory disease, the contribution of various muscles and muscle groups is of considerable interest. The older observers, notably Keith (1), based their conclusions of the contribution of the various muscles to respiration on two types of study. One was the anatomical position of the muscle in relation to the direction of the fibers and the origin and insertion of the muscle. The other was the study of muscle function by observation or palpation of the contracting muscle. So influential has been the classical treatise of Keith that modern textbooks (2) base their teaching of respiratory muscle function on this work. The observations of recent years have consisted mainly of studies of electromyography—Campbell (3), Campbell and Green (4), Lewis (5), Dickinson (6) and Floyd and Silver (7). While these investigators have recognized the limitations of electromyography, such as the uncertainties when using surface electrodes in recognizing electrical activity of a deep muscle below a superficial muscle and the uncertainty of determining the exact location of the active tip of the less popular needle electrodes, much useful information has been obtained. In particular, electromyographic studies have revealed the association of electromyographic activity with muscle function during quiet and deep breathing, in hyperventilation, maximum inspiratory and expiratory effort against pressure, coughing, straining and in maintenance of posture. One muscle whose participation in respiration cannot be easily evaluated by electromyography is the diaphragm. As will be shown later, it is this muscle whose participation in respiration can be particularly evaluated by the present study of paraplegics.

The work reported here consists of a study of a group of patients who have varying degrees of muscle paralysis caused by a traumatic lesion of the spinal cord. The characteristic feature of this group is that the lesion is complete and well defined, with absence of ventilatory function below the lesion and normal function above. By selection of patients with lesions at various spinal segments and testing with standard pulmonary function tests, it has been possible to evaluate the effect on breathing caused by functional elimination of the respiratory muscles below any spinal level. For patients with a "low" lesion, that is, in the lower lumbar region, the only loss of function of body muscles is that of the pelvic floor. With "high" lesions in the lower cervical region the only effective muscle remaining for respiration is the diaphragm. This method of evaluating the role of various groups of muscles as contributors to respiration differs from the usual methods of studying pulmonary muscle function mentioned above and provides supplementary information not obtainable by other methods.

METHODS

The patients studied were selected from approximately 400 patients of the Paraplegia Service of the Long Beach Veterans Administration Hospital. This particular service has special facilities for care, treatment, research, social and economic management of this type of patient. From this group 64 patients were selected for study. The selection was determined by completeness of the spinal cord lesion, freedom from complicating factors such as muscle spasm, willingness of the patient as a cooperative subject and absence of other respiratory disorders than that caused by the lesion. The patients were mostly in the 20 to 40 year age group. They were all men and were the victims of the accidents listed in Table I, the majority of these being automobile accidents. Before injury these men were in excellent health, were muscular and most were engaged in outdoor work or outdoor activities. Between the time of the injury and the time of measurement, weight loss occurred of approximately 5 to 20 per cent. The tests were made at a time after injury varying from 4 months to 10 years. The time interval between injury and testing, together with the ages of the subjects, is shown in Figure 1.

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TABLE I

Number of patients tested and cause of accident

Cause of accident	No. patients	Per cent
Automobile	34	54.0
Gunshot	8	12.8
Diving	7	11.4
Falls	5	8.0
Logging	2	3.1
Airplane	2	3.1
Train	2	3.1
Mining	1	1.5
Ship explosion	1	1.5
Rupt. intervertebral disc	1	1.5
Total	63	100.0

The patients were divided into three groups according to the level of the spinal cord lesion. Group I included 21 patients with lumbar lesions; Group II, 14 patients with thoracic lesions; and Group III, 29 patients with cervical lesions. The extent of muscle paralysis of the three groups is shown in Figure 2. The members of Group I with lumbar lesions had paralysis of the pelvic floor and the lower limbs. If the muscles of the pelvic floor are excluded as contributing to respiration, then these men had respiratory functioning musculature which was normal. The members of Group II had lesions in the thoracic region. These extended from the first to the tenth thoracic level. Patients of this group were more difficult to obtain because injuries to this region of the back were fewer due to mechanical protection of the rib cage, and well defined spinal transections were less likely to occur. Group III includes the patients with lesions from the fourth to the eighth cervical segment. A few of those with the higher lesions had limited use of the arms and were unable to perform the exercise test described later. Patients in this group, as shown in Figure 2, had paralysis of all the muscles of respiration except the accessory muscles and the diaphragm.

A group of 17 normal men served as controls. These men were staff physicians, residents, interns, aids and technicians. Their age distribution is shown in Figure 1. These men were tested in the same manner as the patients except that the arterial blood studies were omitted.

There were four groups of tests made including *a*) spirometry, *b*) residual volume and nitrogen clearance during oxygen breathing, *c*) blood gas analysis and *d*) an exercise tolerance test. *a*) The spirometry tests consisted of a measure of inspiratory vital capacity, expiratory reserve volume, and maximum breathing capacity. The inspiratory vital capacity as defined here is the increase in lung volume caused by a maximal forced inspiration following a maximal expiration. The Collins 13 liter spirometer was used. Four determinations of inspiratory vital capacity were made and the largest value chosen. Nine measurements of expiratory reserve volume were made and the mean value used. Two measurements of the maximum breathing capacity were made and the highest values used. *b*) Residual volume was measured by the open-circuit method of Darling, Cournand, and Richards (8). *c*) Arterial blood was drawn and analyzed for oxygen, carbon dioxide content and oxygen capacity using the Van Slyke manometric apparatus. Arterial pH was determined with the Cambridge glass electrode. *d*) An exercise test was devised which consisted of raising from the supine position two 5 pound hand weights, one in each hand, from the side to above the head. This weight lifting was performed once every two seconds for five minutes. This type of test was used because an arm movement exercise was the only type of exercise capable of being performed by these patients. Oxygen consumption rate, carbon dioxide production rate, respiratory quotient, ventilation rate, tidal volume, ventilation coefficients, and alveolar (end-tidal) carbon dioxide tensions were measured in a five minute resting period before the exercise, during the five minutes of exercise, and in a five minute postexercise period. Respiratory dead space was computed using the Bohr formula (9) and the values of CO_2 in expired and alveolar air. A mouthpiece with a Douglas valve was used with

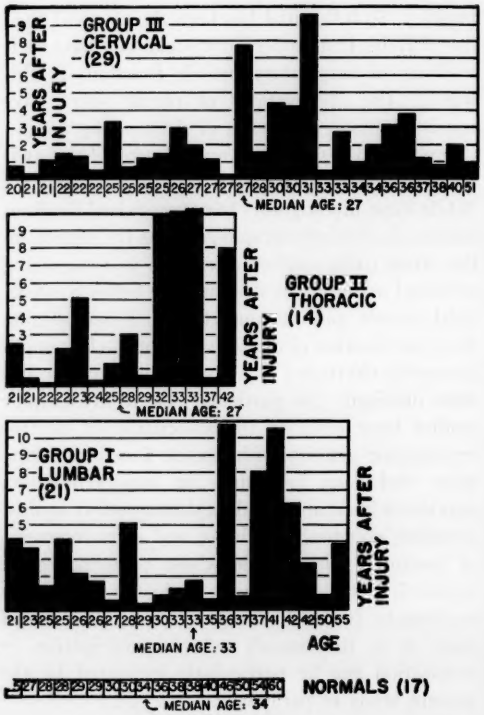

FIG. 1. AGE DISTRIBUTION, TIME INTERVENING BETWEEN INJURY AND TESTING AND NUMBER OF INDIVIDUALS (IN PARENTHESES) IN EACH GROUP

The age distribution of the 17 normal men of the control group is given at the bottom of the figure.

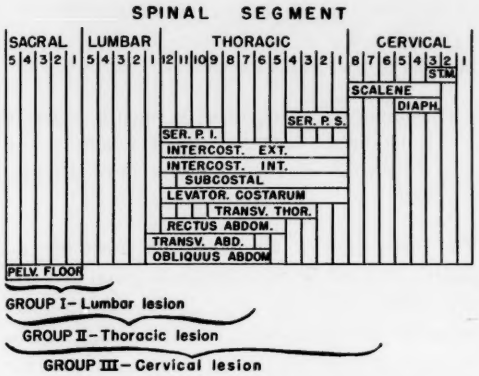

FIG. 2. ANATOMICAL EXTENT OF MUSCLE PARALYSIS IN EACH OF THE THREE GROUPS TESTED

a 120 liter spirometer for collecting expired air. Gas analyses were made with the Scholander (10) apparatus. End-tidal (alveolar) carbon dioxide was measured in two ways: *1*) by collecting small aliquots of end-tidal air from approximately 10 exhalations beyond the expiratory value of the Douglas mouthpiece, or *2*) passing the expired air through the seven-eighths inch tube of the Model 16 Liston-Becker carbon dioxide analyzer. The gain control and the zero control of the infra-red carbon dioxide analyzer were set immediately before the test with two carbon dioxide air mixtures of known composition. A calibration curve was made for the instrument in which instrument reading was plotted against alveolar air CO_2 determinations using the gas collection technique with chemical gas analysis. In the majority of determinations (approximately 95 per cent) both infra-red CO_2 analysis and chemical analysis were made. The chemical gas analysis values were considered more reliable and were chosen in preference to those of the CO_2 analyzer. The two (chemical and infra-red) differed only slightly with approximately 80 per cent of the two determinations differing by only plus or minus 1 mm. in the computed P_{ACO_2}.

RESULTS

Vital capacity

The inspiratory vital capacity of the control group was determined and from the data a linear regression formula relating vital capacity to age was derived. The formula is:

Inspiratory vital capacity = 2.857 − 0.101 × age ± 0.335 (liters, 37° C., ambient pressure saturated with water vapor).

Another prediction formula was the "height formula" given by Baldwin, Cournand, and Richards (11). Using these two prediction formulas, the predicted vital capacity of each patient was determined and from their measured vital capacity, the percentage of the predicted value was obtained. These percentages with standard deviations are given in Table II. For each group and for each prediction formula the probability of a significant difference from normal was determined using the Fisher test. In the table, if a group is significantly different from normal, it is designated S; if not significantly different, NS. It was considered significantly different if the probability of being different exceeded 99 per cent, or the probability, p, of nonsignificance was less than 0.01.

The patients of Group I (lumbar lesion) had vital capacities which did not differ significantly from normal. Group II patients (thoracic lesion) had vital capacities significantly smaller (82.9 per cent) than our control group but not significantly less than predicted by the Baldwin height formula. Group III patients had vital capacities significantly less than normal (approximately two-thirds of normal). Their vital capacities were surprisingly high considering the extensive paralysis and the availability for breathing of only one important respiratory muscle, the diaphragm.

TABLE II

Vital capacity of the three groups of paraplegics compared with the normal standard and a group of 17 normal individuals tested with the same procedure as the paraplegics

	Percentage values of the predicted (mean and S. D.)	p*
	%	
Group I (lumbar lesion)		
West formula†—22	99.4 ± 18.8	
Height formula‡—22	100.5 ± 18.3	NS
Normal group	94.0 ± 15.9	NS
Group II (thoracic lesion)		
West formula—16	90.9 ± 13.8	
Height formula—16	92.4 ± 19.6	NS
Normal group—17	82.9 ± 17.0	S
Group III (cervical lesion)		
West formula	70.3 ± 12.7	
Height formula	71.8 ± 13.1	S
Normal group	64.9 ± 10.5	S

* The letters in this column are used to indicate a significant difference (S) or no significance (NS) for a probability of being significantly different greater than 99 per cent; *i.e.*, p less than 0.01.

† West formula, $VC/M.^2 = 2.50$ liters.

‡ Height formula, VC = (27.63 − 0.112 × age) (Ht. in cm.) (ml.).

TABLE III

Variation of expiratory reserve volume/vital capacity percentage with level of spinal injury

VC = EC + IC*		p†
	%	
EC/VC ratio		
Group I—22	19.0 ± 7.1	NS
Normal group—17	17.6 ± 5.2	
Group II—16	19.7 ± 4.0	NS
Normal group—17	19.5 ± 5.2	
Group III—24	8.9 ± 5.3	S
Normal group—17	18.9 ± 5.9	

* Vital capacity (VC) equals expiratory capacity (EC) plus inspiratory capacity (IC).
† NS, no significance; S, significant difference.

Expiratory reserve volume/inspiratory vital capacity ratio

It is to be expected that, with lesions of the spinal cord in the thoracic or cervical segments, the expiratory effort would be more likely to be impaired than the inspiratory effort presuming that the abdominal muscles are the powerful expiratory muscles. Considering the two components of the vital capacity as the inspiratory capacity and the expiratory reserve volume, and defining the expiratory reserve volume as the decrease in lung volume caused by a forced expiration starting from the resting expiratory level, one would expect that with abdominal muscle paralysis the expiratory reserve volume would be decreased more than inspiratory capacity. The ratio of expiratory reserve volume to vital capacity expressed as percentage is given in Table III with the probability of there being a significant difference between paraplegic and control groups. For the paraplegics with lumbar and thoracic spinal cord lesions there was no significant difference between normals and paraplegics. With cervical lesions, however, the expiratory reserve volume percentage was reduced significantly to approximately one-half.

Maximum breathing capacity

In Table IV are given the values of maximum breathing capacity expressed as percentages of the standard predicted values. These standards are those of Motley (12) and Baldwin, Cournand, and Richards (11). The maximum breathing capacity of the control group of normal individuals was determined and a regression formula derived as a function of age. This formula is:

Maximum breathing capacity/square meter of body surface = $92.9 - 0.654 \times \text{age} \pm 13.6$ (liters, 37° C., ambient pressure saturated with water vapor).

The percentage of the maximum breathing capacity predicted by this formula is given in Table IV. The probability of a significant difference from the predicted, with $p < 0.01$, is indicated by S (see vital capacity). For Group I (lumbar lesion) the maximum breathing capacity did not differ significantly from the normal predicted values of the Baldwin formula or our control group. However, compared with the Motley formula (which has higher values than the Baldwin or our control group) the Group I paraplegics were significantly lower. For Groups II and III the maximum breathing capacity values are all significantly lower than predicted.

TABLE IV

Maximum breathing capacity as percentage of standard predicted values and predicted values from control group

		p*
Group I—21		
Lumbar lesion		
Motley standard†	84.4 ± 25.3	S
Baldwin standard‡	94.5 ± 28.6	NS
Control group§	96.7 ± 28.6	NS
Group II—16		
Thoracic lesion		
Motley standard	61.0 ± 15.6	S
Baldwin standard	70.4 ± 17.8	S
Control group	71.1 ± 15.6	S
Group III—24		
Cervical lesion		
Motley standard	48.6 ± 15.4	S
Baldwin standard	56.3 ± 17.4	S
Control group	56.9 ± 17.4	S

* S, significant difference; NS, no significance.
† Motley standard: Maximum breathing capacity per M.² equals 97 minus age/2 (12).
‡ Baldwin standard: Maximum breathing capacity per M.² equals 86.5 minus 0.522 times age (liters B.T.P.S. per minute (11).
§ Control group: Maximum breathing capacity per M.² equals 90.8 minus 0.68 times age.

Residual volume

The residual volume in liters B.T.P.S. per square meter of body surface for the paraplegics compared with normal controls is shown in Figure 3. A linear regression formula for the normal controls was computed and found to be:

Residual volume = 0.64 + 0.0073 × age ± 0.16 (liters per square meter body surface, 37° C., ambient pressure saturated with water vapor).

The regression line corresponding to this formula is shown as the center line of Figure 3 with the limits of standard error of estimate forming the two outer lines. On the same figure individual values of residual volumes for the three groups of paraplegics are indicated by points. The majority of the paraplegics have residual volumes greater than normal as is obvious from the location of the points on the graph. That they are significantly greater is shown by computation of the mean of each group and the mean of a normal group of the same age. The means are shown in the small inset table of Figure 3.

The residual volume/total capacity ratio for patients and normal controls of the same age is given in Table V. This ratio for the control group of 17 normal individuals was found to be:

RV/TV × 100 = 19.50 + 0.201 × age ± 3.50 (per cent).

For each of the three groups of patients the mean ratio for a group of normals for the same age was obtained using this formula. This mean ratio for each normal group is given in Table V, the mean values for each normal group being slightly different because of differences in the mean age of each group. The residual volume/total ca-

TABLE V

Percentage ratio of residual volume/total capacity (RV/TC) for the three groups of paraplegics

		p*
	%	
Group I (lumbar lesion)—22	32.7 ± 8.0	S
Normal controls—17	26.3 ± 3.5	
Group II (thoracic lesion)—16	36.0 ± 9.2	S
Normal controls—17	25.0 ± 3.5	
Group III (cervical lesion)—24	41.3 ± 6.1	S
Normal controls—17	25.4 ± 3.5	

* Significant difference.

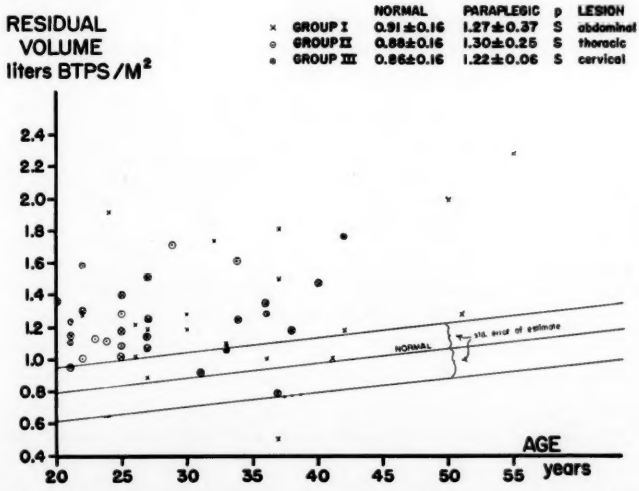

FIG. 3. VARIATION OF RESIDUAL VOLUME* PER SQUARE METER OF BODY SURFACE WITH AGE FOR NORMAL CONTROLS AND THREE GROUPS OF PARAPLEGICS

The center line is the linear regression line of the controls and the two outer lines are the limits of the standard error of estimate. Residual volume is expressed as liters at 37° C., ambient pressure, saturated water vapor.

TABLE VI

Arterial blood gases and pH for the three groups of paraplegics *

		I Lumbar	II Thoracic	III Cervical
O_2 content	$\bar{x}$	17.6	17.2	17.5
	σ	1.5	1.3	1.2
O_2 capacity	$\bar{x}$	18.8	18.3	18.6
	σ	1.7	1.7	0.9
Per cent saturation of hemoglobin	$\bar{x}$	94.4	95.4	95.8
	σ	3.0	2.4	1.1
CO_2 content	$\bar{x}$	48.3	49.3	48.3
	σ	3.6	4.5	2.5
pH	$\bar{x}$	7.40	7.42	7.38
	σ	0.07	0.09	0.08

* Oxygen content, oxygen capacity and carbon dioxide content are given as ml. of each gas STPD (0° C., 760 mm. pressure, dry) in 100 ml. of whole blood. $\bar{x}$ is the mean value and σ the standard deviation.

pacity ratio is significantly greater than normal controls of the same age as is shown in Table V. The higher the lesion the greater is this ratio. The total capacity is the sum of vital capacity and residual volume.

Blood gases

The arterial blood oxygen and carbon dioxide, as well as arterial pH, are shown in Table VI. Values given are the means and standard deviations for each group. These values are normal.

Exercise test

In Table VII are given the data for the five minute weight lifting exercise test. The data in rows opposite "rest" are those collected in a five minute preexercise resting period. The postexercise period immediately following the exercise period lasted five minutes. The values in the table are the means ($\bar{x}$) and the standard deviations (σ) for the individuals of each group. With this "mild" exercise the oxygen consumption rate ($\dot{V}_{O_2}$) in ml., 0° C., 760 mm. Hg pressure, dry, per square meter of body surface per minute and the ventilation rate ($\dot{V}_E$) in liters, 37° C., ambient pressure, saturated with water vapor per square meter of body surface per minute, approximately doubled. As is characteristic of exercise, there was a slight rise in respiratory quotient and in ventilation coefficient ($\dot{V}_{O_2}/\dot{V}_E$).

Respiratory dead space

The respiratory physiological dead space values as determined by the Bohr formula (9) and using

TABLE VII

Ventilation data of normal controls and three groups of paraplegics for a five minute weight lifting exercise test *

		Normal N 15		Lumbar Group I 15		Thoracic Group II 11		Cervical Group III 14	
Number		$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ
O_2 consumption rate (OCR) ml. STPD/min. M^2	Rest	124	18	121	14	124	14	132	22
	Ex	251	40	259	32	274	27	211	36
	Post Ex	141	19	153	26	155	13	145	32
CO_2 production rate (CPR) ml. STPD/min. M^2	Rest	95	11	92	13	98	11	108	20
	Ex	210	37	200	25	227	21	180	31
	Post Ex	120	16	131	25	130	16	146	30
Respiratory quotient	Rest	77	5	76	9	79	7	82	5
	Ex	84	5	77	8	83	6	85	9
	Post Ex	85	5	85	11	83	10	83	6
Ventilation rate (V_E) BTPS/min. M^2	Rest	3.12	0.42	3.67	0.17	3.50	0.64	3.81	0.80
	Ex	6.17	0.80	6.35	0.73	6.94	0.32	5.99	0.98
	Post Ex	3.85	0.35	4.88	0.96	4.46	0.55	4.30	0.75
OCR/V_E	Rest	39.8	3.5	33.4	3.4	35.3	5.9	34.7	4.2
	Ex	40.9	5.8	41.0	4.5	40.1	4.5	36.7	6.7
	Post Ex	37.0	4.9	31.5	4.4	34.3	3.4	33.8	5.0
O_2 debt		214	13	198	20	148	64	108	60

* Each test was divided into three successive periods including a five minute preexercise (resting) period, the five minute exercise period, and a five minute postexercise period.

TABLE VIII

Respiratory dead space (V_D) (in ml. per square meter of body surface) and the dead space/tidal volume ratio (V_D/V_T) expressed as percentage *

		Before exercise		During exercise		After exercise	
		V_D (*ml.*)	V_D/V_T (%)	V_D (*ml.*)	V_D/V_T (%)	V_D (*ml.*)	V_D/V_T (%)
Normal group (N = 14)	$\bar{x}$	87.9	31.6	110.0	23.3	87.7	24.8
	σ	9.2	3.1	25.8	7.5	17.3	5.9
Group I (N = 15)	$\bar{x}$	93.9	31.8	121.8	24.1	96.1	26.2
	σ	17.2	5.5	26.9	5.9	25.3	5.9
Group II	$\bar{x}$	98.8	29.5	94.1	17.2	97.1	27.2
	σ	20.7	5.2	20.3	4.8	16.2	4.8
Group III	$\bar{x}$	100.1	28.4	86.4	21.1	94.8	25.9
	σ	21.5	5.9	16.8	6.5	26.0	7.4

* $\bar{x}$ is the average for the group and σ is the standard deviation. N is the number of individuals in the group.

carbon dioxide analyses of end-tidal (alveolar) air and expired air are given in Table VIII with values of the dead space/tidal volume ratio. For the normal control group and the patients with lumbar spinal transections the dead space volume increased with exercise and the dead space percentage of the tidal volume decreased, a finding in agreement with previous investigators (9). However, for the patients with thoracic lesions (Group II) the dead space decreased slightly (but insignificantly) with exercise, while for patients with cervical lesions the dead space fell more. If a statistical comparison is made of the dead space change with exercise and Group I, or the normal control group, compared with the Group III (cervical lesion), a statistically significant difference between the two groups ($p < 0.001$) exists.

DISCUSSION

In the management of paraplegics, respiratory dysfunction is not as serious a clinical problem as bowel and bladder regulation, decubitus ulcers and urinary infection. In tetraplegics, respiratory dysfunction occurs early in the post-traumatic phase when a tracheostomy may be needed and later if an intercurrent upper respiratory infection develops. With an upper respiratory infection, difficulty is experienced by the patient due to failure of active expectoration. Removal of tracheobronchial secretions can be aided if necessary by postural drainage, splinting of the abdomen and liquefaction of bronchial secreta with expectorants. Usually, however, for routine activity of the tetraplegic the existing, but impaired, cough mechanism with tracheobronchial ciliary function is adequate.

It is evident from the data on vital capacity, maximum breathing capacity, exercise ventilation and blood gases that the ventilation of the paraplegics with lumbar lesions is essentially normal. This is readily explainable since the muscular paralysis of these patients is limited to that of the lower extremities and pelvic floor while the respiratory musculature is functioning normally. The patients of Group III with lower cervical lesions have paralysis of abdominal and intercostal muscles, and the sole remaining effective respiratory muscle is the diaphragm. In spite of this extensive respiratory muscle paralysis, the entire ventilation as performed by this one remaining functional muscle is surprisingly effective. Approximately, for this group, the vital capacity is two-thirds of normal and the maximum breathing capacity one-half of normal.

The expiratory reserve volume is the decrease in volume of the lung caused by a forced expiration commencing from the resting expiratory level. For this forced expiration the expiratory muscles are active participants. Since with thoracic spinal cord lesions part of the expiratory muscles are paralyzed, and with cervical lesions all are paralyzed, it is to be expected that the expiratory reserve volume would be decreased. It was found that with the normal control group and Group I (lumbar lesion) and II (thoracic lesion) the ex-

piratory reserve volume percentage of the vital capacity was approximately the same, *i.e.*, approximately 17 to 19 per cent. With Group III (cervical lesions) the expiratory reserve volume was approximately 9 per cent of the vital capacity, a reduction from the normal value of approximately one-half. The surprising finding is that these patients with no functional expiratory muscles can exhale forcibly this much air from the resting expiratory level. The reason for this forcible exhalation is not readily apparent. Possibly the shoulder girdle muscles participate in exhalation more than is generally believed. Another explanation is that the residual volume of these patients is increased approximately 500 ml., as is shown in Figure 3, which would indicate that they are breathing from a more inspiratory chest position than normal. The term resting expiratory position of the chest, although it implies minimal or no diaphragmatic muscle tonus, does not necessarily mean that such tonus is absent. The work of Culver and Rahn (13) and the earlier work on pressure breathing (14) have provided evidence that at the resting expiratory level there may be partial contraction of the respiratory musculature, and it is possible that in the abnormal condition of respiratory muscle paralysis where the diaphragm is the only muscle of respiration, an increased diaphragmatic tone can occur at the resting expiratory level. This would permit an expiratory volume of air to be exhaled from the resting expiratory level simply by relaxation of diaphragmatic tonus. Although this might explain the unanticipated high value of the expiratory reserve volume, it does not explain why the residual volume is elevated. The residual volume was found to be elevated in all of the three groups of paraplegics and did not seem to be dependent on the extent of respiratory muscle paralysis. The only paralysis common to the three groups was paralysis of the lower limbs and pelvic floor muscles. In addition all the patients led a sedentary or supine type of living while the controls were actively working. These differences do not afford a simple explanation for the increased residual volume. One explanation which is proposed reservedly is that the pelvic floor muscles support the abdominal viscera. Paralysis of these muscles withdraws some support from the abdominal viscera causing the diaphragm to descend to increase residual volume. If this occurs, it imparts to the pelvic floor more of a supporting role for the viscera than is generally believed to occur.

The values observed for blood gases and ventilation studies during exercise were essentially the same for the patients as the controls. These observations indicate that the extensive paralysis of respiratory muscles causes no serious interference with blood gas exchange in the lung and that a mild exercise (equal to approximately two and a half times the resting oxygen consumption rate) can be performed without ventilatory difficulty. The fact that oxygen consumption rate of the paraplegics being essentially the same as the controls suggests that widespread denervation of the skeletal muscle does not reduce oxygen demand of the tissues. This is an observation of theoretical interest in the control of metabolism and the needs for homothermy.

In the response to mild exercise a normal individual increases his ventilation more by deep breathing than by increased respiratory rate. Associated with this increased tidal volume the respiratory physiological dead space as measured by the CO_2 method and using the Bohr formula is increased while the dead space/tidal volume fraction is reduced (9). This response with values which were similar to those in the literature were found for the individuals of the normal control group and those of Group I (lumbar lesions). With Group II (thoracic lesions) the mean respiratory dead space for the group fell slightly, the decrease being of questionable significance. With Group III, however, the respiratory dead space was reduced considerably more with exercise, the mean value decreasing from 100.1 to 86.4 (ml., 37° C., ambient pressure, saturated with water vapor per square meter of body surface). If a statistical comparison of the changes in dead space before and during exercise is made for Group I (or the normal control group) and Group III (cervical lesions) a significant difference ($p < 0.001$) for the dead space change of the two groups is found. The reason for this reduction in dead space with exercise for the Group III paraplegics is not clear. Some individuals of this group breathed irregularly and erratically during exercise. This group, more than the others, during

exercise increased their respiratory rate and their tidal volume increase was less. This irregular breathing increased the difficulty of sampling end-tidal air. However, large errors in this sampling probably did not occur since the following end-tidal mean values of CO_2 (dry, volumes per cent) before (B) and during (D) exercise were obtained: controls, (B) 5.39, (D) 5.43; Group III, (B) 4.75, (D) 4.75. The problem of measuring respiratory dead space by the CO_2 method is one on which some of the most heated controversies of exercise physiology have been waged and many of the problems are still unsettled (9). It is concluded from the observations here reported that with diaphragmatic breathing of cervical paraplegics, physiological dead space does not increase but may decrease with exercise. This might be due to the fact that diaphragmatic breathing moves the lower parts of the lung adjacent to the diaphragm while the upper part of the lung containing the large airways, and hence the dead space, remains relatively immobile.

SUMMARY

Pulmonary function studies have been made on a group of 63 paraplegic patients with complete spinal transections and a group of 17 normal controls. The patients were divided into three groups: *1*) those with lumbar spinal lesions who had paralysis of the lower limbs and pelvic floor but no paralysis of the muscles of breathing; *2*) those with thoracic spinal lesions with paralysis of the lumbar muscles and lower intercostals; and *3*) those with lower cervical spinal lesions with paralysis of all muscles of breathing except the diaphragm and the accessory muscles of respiration. Measurements were made of vital capacity and components, residual volume, maximum breathing capacity, arterial blood gases and pH, and ventilation during exercise. It was found that the vital capacity and maximum breathing capacity of patients with lumbar lesions were normal, while the group with lower cervical lesions had a vital capacity approximately two-thirds of normal and a maximum breathing capacity of one-half of normal. Paraplegics with thoracic lesions had vital capacities and maximum breathing capacities between the values for the cervical and lumbar lesions groups. The resting expiratory reserve volume expressed as a percentage of the vital capacity was below the normal percentage only for the group of paraplegics with cervical lesions. For this group the percentage value was one-half normal, which is higher than was anticipated for patients with only inspiratory muscle function. Residual volumes of all groups were significantly higher than the normal group. Arterial blood gases of all groups were normal and the ventilatory response to exercise for all groups of paraplegics did not differ from normal.

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